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Carcinoma

PRINCIPAL INVESTIGATOR: Patnala Achary, Ph.D.

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine  
Bronx, New York 10461

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<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Albert Einstein College of Medicine Bronx, New York 10461  <b>E-MAIL:</b> achary@aecom.yu.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
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<b>13. ABSTRACT (Maximum 200 Words)</b> The clinical outcome is generally positive for patients with node-negative breast carcinoma. In about 13% of those patients, however, the disease spreads, and they are at risk of death. Our goal is to develop DNA markers that could be reliably used to identify the ductal mammary carcinomas that are prone to develop metastasis. We compared DNA from normal cells and metastatic cells and also primary tumor cells and metastatic cells by representational difference analysis (RDA) method. We have isolated 15 metastasis associated DNA sequences (MADS), of which 3 were found be associated with metastasis in breast cancer patient samples other than the index case that was used in RDA experiments. Screening of primary tumors using MADS as fluorescence in situ hybridization (FISH) probes showed that MADS-IX is homozygously lost in some of the tumor cells (3/50) of a primary tumor that had positive lymph nodes where as it is not homozygously lost in any of the tumor cells (0/50) in the primary tumor that did not develop metastasis. Screening of additional cases is underway to determine if these results will be statistically significant.				
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### 3. Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-11
Key Research Accomplishments.....	11-12
Reportable Outcomes.....	12-14
Conclusions.....	14-15
References.....	15
Appendices.....	I-IX

#### **(4) Introduction:**

The clinical outcome is generally positive for patients with node-negative breast carcinoma (i.e., those who do not have detectable metastases in the lymph nodes) who have been treated with surgery or surgery and radiation therapy. In about 13% of the patients, however, the disease spreads, and they are at risk of death (Lewis and Conry, 1992; Scorilas et al., 1993; Fisher et al. 1997). Genetic tests capable of identifying patients at risk for metastatic spread and/or better treatment targeted to eradicate metastatic tumor deposits could have a dramatic impact on the overall survival of these patients. The development of a panel of molecular genetic markers for identifying the 13% lymph node negative mammary carcinomas that are known to develop metastases (Fisher et al. 1997) would be clearly be of considerable value in indicating those cases in need of early aggressive therapy. There would also be considerable benefit to the 87% of women with mammary carcinomas that are not likely to metastasize by sparing them the considerable physical, mental and financial costs of the treatment.

#### **(5) Body:**

The technical objective of this project is to isolate genes or DNA sequences whose loss represents a step in the acquisition of metastatic property by ductal mammary carcinoma cells. To accomplish this objective we have been using representational difference analysis (Lisitsyn et al. 1993; Li et al. 1997) on normal/primary and metastatic archival tissue samples from patients with breast carcinoma. Attaining this aim will result in a panel of molecular genetic markers that could be used for differentiating with high sensitivity and reliability ductal mammary carcinomas that are prone to developing metastases from those that will not.

Statement of Work as proposed in the grant application (3 years)

Task. 1: Isolation of differential sequences specific to breast metastasis using RDA.

- i. Collection of additional tissues of ductal mammary carcinoma (DMC) and continuation of ongoing RDA product characterization (Months 1-12)
- ii. Confirmation of histopathology of tissues before LCM (Months 1-24)
- iii. LCM of 30 cases of DMC tissues with normal, primary and metastasis cell components (Months 2-30)
- iv. RDA of 30 cases of DMC (Months 1-14)

Task. 2: Characterization of the differential RDA products.

- i. Cloning of RDA products isolated from the loss side (Months 1-18)
- ii. Sequencing and Southern blot analysis of the clones (Months 1-20)
- iii. Homology search of positive clones (Months 2-28)
- iv. Northern blot analysis of the analysis of significant clones from the past and present RDA experiments (Months 6-25)
- v. Isolation of complete gene sequences (Months 6-25)

Task. 3: Further analysis of candidate genes of metastasis.

- i. Screening the metastasis-specific genes on at least 120 DMC cases and statistical analysis (Months 12-30)



- ii. *In vitro* and *in vivo* functional assays of metastasis (Months 12-30)
- iii. Antisense oligonucleotide-mediated disruption of mRNA translational studies (months 18-34)
- iv. Compilation of data for the submission of final grant report (Months 33-36).

### **Third Year Progress Report:**

The following is the progress of the work done so far in the third year (Please note that we are now named markers associated with metastasis as Metastasis Associated DNA Sequences [MADS] instead of Metastasis Associated Gene Sequences [MAGS]):

#### **Task 1:**

All the proposed experiments have been completed. However, we found that the remaining archival tumor tissue samples (paraffin embedded tumor tissue samples) were not suitable for microdissection by Laser Capture Microdissection (LCM). Though we were successful in performing Single Cell Microdissection (SCM) on some of the cases, unfortunately the DNA isolated from these microdissected cells was degraded and not suitable for Representational Difference Analysis (RDA) experiments.

Besides, the 12 RDA experiments we have performed so far yielded about 1600 clones. Of them, we randomly selected only 700 for preliminary characterization. Characterization of RDA clones (MADS) took most of our time. However, if time permits, we wish to characterize the remaining clones instead of performing additional RDA experiments, to isolate additional Metastasis Associated DNA sequences (MADS).

#### **Task 2:**

As mentioned previously, we have isolated 11 candidate MADS. From the remaining RDA experiments we isolated 4 additional novel MADS using our "total probe" screening method. From each of the RDA experiments (loss side) except for the first experiment (in which 100 clones were randomly selected), 50 clones were selected and transferred onto separate nylon membranes. In this way we prepared 8 membranes from 8 RDA experiments using tumor tissue samples and 4 membranes from RDA experiments using 2 pairs of cell lines. The DNA from the 11 MADS (isolated from the first RDA) were mixed in equal amounts and used as a 32p-dCTP probe (Total probe) on each of the 12 membranes. We picked up 47 clones that did not hybridize with the total probe. We believe that these sequences are novel (not present in the 11 MADS mixture). Similar to those differential sequences that were isolated from the first RDA, these 47 candidate clones were sequenced. Sequencing revealed additional 4 groups of MADS. Thus bringing the total number of MADS to 15.

The sequencing of 4 new MADS showed the following homology in the gene banks:

MADS-XII: Size: 125bp; 96% homology with BAC clone RP11-651C2 on chromosome 4.

MADS-XIII: Size: 212bp; 99% homology with BAC clone RP11-452C13 on chromosome 7.

MADS-XIV: Size: 231bp; 92% homology with BAC clone AC000119 on chromosome 7.

MADS-XV: Size: 172bp; 95% homology with BAC clone AL590825 on chromosome 6.

### Isolation of complete MADS:

Generally RDA recovered size of differential sequences are in the range of 100-350bp. For example, MADS-IV is only 185bp. Since MADS-IV seemed to be a novel sequence without any homology in the gene banks, in order to obtain partial/full-length sequences of MADS-IV, we used human mammary cDNA library screening and Inverse-PCR (I-PCR) methods. The reason behind these 2 approaches is to obtain complete gene sequence or reasonably long enough sequence so that we can use these MADS for transfection studies as well as use as FISH probes (more than 1Kb size of DNA sequence is required to use as FISH probe) to screen normal and tumor cells simultaneously on primary and metastatic tumor tissue sections of patient samples. However recently we changed our approach in this regard. We are planning to use BACs containing MADS directly as FISH probes and also transfect retrofitted BACs (please see under task 3 and also attached R-21 grant application) containing MADS into highly metastatic human breast carcinoma cell lines to evaluate their metastatic potential. This strategy we believe will obviate the antisense experiments we proposed under task 3, for evaluating the metastatic potential of MADS.

### **Task 3:**

### Further characterization of promising MADS:

#### MADS-IV:

As mentioned earlier MADS-IV is a novel sequence showing no homology in the NCBI gene banks. We showed that MADS-IV is missing in 4 cases (including the index case used for RDA). RT-PCR results of MADS-IV using total RNA from normal and tumor tissue samples revealed that it is an expressed sequence. To confirm if this sequence is tissue-specific, we screened multiple tissue RNA Master Blots<sup>TM</sup> (CLONTECH Laboratories, Inc.) using MADS-IV as a  $\alpha^{32}$ p-dCTP probe. Results revealed that MADS-IV is not tissue-specific (expresses in mammary gland and also in all the other human tissues, but not in non-human RNA or cDNA samples), indicating that MADS-IV is a part of a human gene that transcribes commonly in human tissues (Fig. 1). This reiterates the fact that this MADS could, possibly be a part of a functional gene involved in breast tumorigenesis/metastasis or both.

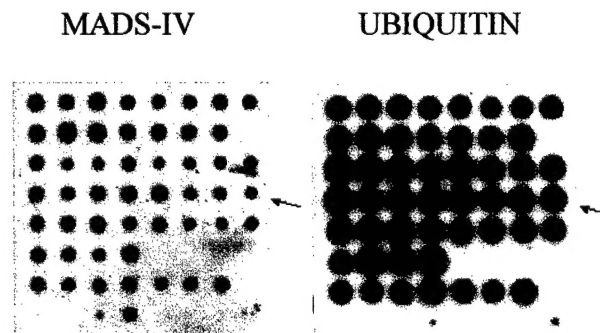


Fig. 1. Results of Northern hybridization of RNA Master Blot (CLONTECH Laboratories, Inc.) using MADS-IV as probe. It showed expression in all of the human tissues, but not in non human RNA or

cDNA (synthetic poly r(A), yeast tRNA or total RNA, or C<sub>0</sub>t1 DNA) indicating that it is an expressed human gene sequence. No significant change in the intensity of signal was noticed in breast tissue (Arrow) compared to the remaining tissues. Ubiquitin gene was used as an internal control for normalization.

### MADS-XI:

This sequence is a novel dinucleotide repeat sequence (TG/AC) which was found to be missing (partial/complete) in 5 out of 6 metastatic DNA samples in a slot blot. After repeated attempts, recently we are now successful in sequencing the complete MADS-XI (Fig. 2; also please see the attached manuscript by Mukherjee et al, 2002 for details).

**VGATCTTAATCCGGGGAGTGGCGTATGTAGTAGAAGAGTCTGGATTGAGTAGTGTAT  
GGTAACGCCAG (T-G)<sub>5</sub> C (T-G)<sub>4</sub> GTA (T-G)<sub>2</sub> G (T-G)<sub>4</sub> T (T-G)<sub>3</sub> GCA (T-G)<sub>3</sub> G (T-G)<sub>2</sub> TATGG (T-G)<sub>3</sub> G  
(T-G)<sub>3</sub> A (T-G)<sub>4</sub> GTGCG (T-G)<sub>2</sub> AGATACGTGG (T-G)<sub>3</sub> GGG (T-G)<sub>6</sub> G (T-G)<sub>3</sub> G (T-G)<sub>5</sub> GTA (T-G)<sub>2</sub> G (T-G)<sub>3</sub> G V.**

Fig. 2. Complete MADS-XI with unique sequence (bold letters) and enriched with TG repeats; V= vector sequence

### MADS-IX:

To determine if these MADS are indeed associated with metastasis rather than transformation, DNA was used from cells recovered with the LCM method from normal, primary and metastatic tissue samples from 5 additional patients. These samples were screened by PCR using primers designed from different MADS. As shown in the figure 3, MADS-IX was present in normal cell DNA samples of all the 5 patients but the intensity of hybridization was less in the primary tumor cell DNA samples from 2 patients and totally undetectable in the metastatic cell DNA of these two patient samples, suggesting a loss of this gene sequence during progression to metastasis. This analysis revealed that MADS-IX was lost in the transition from normal to primary to metastasis in 2 of 5 cases (3 of 6 cases if the case used in the RDA assay is included). The fact that it was present in primary tumor cells, but missing in lymph node metastatic cell foci strongly suggests that this is a marker for a novel metastasis suppressor gene.

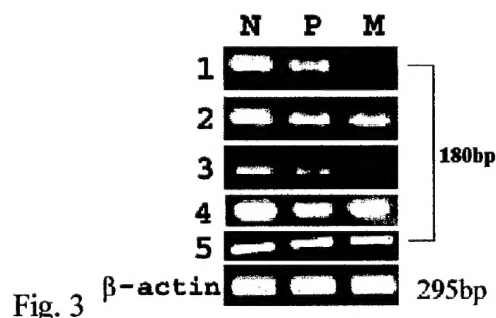


Fig. 3. PCR screening of MADS IX on normal, primary and metastatic cell DNA samples of 5 patients. PCR results showing the target DNA band (180bp) missing in the metastatic cell DNA of patients 1 and 3 (M lane).  $\beta$ -actin as an internal control.

The RH mapping revealed that MADS-IX is localized to a 21cR interval between markers, D10S539 and D10S549, corresponding to human chromosome 10 band q21.1. To determine if this gene is close to PTEN, we screened 4 tumor cell lines and matched normal DNA (the first 3 tumor cell lines had known losses in specific chromosomal regions, the 4<sup>th</sup> cell line had loss of

homozygosity of PTEN gene). PCR screening of MADS-IX and PTEN showed that MADS-IX is present in all the 4 tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937), especially the 4<sup>th</sup> cell line. PTEN is present in 3 cell lines, but missing in the 4<sup>th</sup> cell line, indicating that it is a novel DNA sequence but is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN region (Fig. 4).

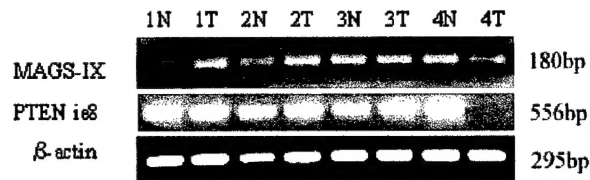


Fig. 4. PCR screening of MADS-IX on normal and tumor DNA samples from breast carcinoma cell lines that losses in specific chromosomal regions (especially the 4<sup>th</sup> which had loss of homozygosity of PTEN gene), showed the presence of MADS-IX in all 4 cell lines. The second row showing the presence of PTEN in the normal cell DNA but missing in the tumor cell DNA of 4<sup>th</sup> cell line (4T lane), indicating that MADS-IX is neither a part of PTEN nor localized in the loss region of 10q which encompasses the PTEN gene.  $\beta$ -actin was used as an internal control.

So far we have been using PCR (Southern slot-blot, where not possible) methods for the characterization of all the MADS on microdissected normal, primary and metastatic tumor cell DNA samples. One of the main problems we encountered was the inconsistency in the PCR results. We had to repeat the PCR experiments several times to confirm the absence/presence of the MADS (partial or complete) in a given patient sample. The partially appearing PCR bands are most of the times confusing because they could be partial loss or a possible contamination from few stromal (normal) cells during microdissection. We therefore proposed to screen the patient samples by FISH. This method we believe is more reliable method to differentiate the archival tumors that did metastasize from those which did not. Since normal and tumor cells are present side by side in a primary tumor sample, we proposed that these markers should be present in normal cells and missing (one allele or 2 alleles) in the tumor cells. In these attempts we used MADS-IX as a FISH probe. Generally, FISH requires larger sized probes (DNA fragment: 1-1000kb). Since the size of the MADS-IX is only 180bp, we designed primers from the BAC clone that has homology with MADS-IX, and isolated a DNA fragment sized 2 Kb encompassing the MADS-IX by long-accurate PCR using normal human genomic DNA as the template. This 2 Kb MADS-IX is used as a FISH probe to localize on human metaphase chromosomes to determine that MADS-IX is a human sequence and not an artifact. We labeled MADS-IX with spectrum green (Vysis) and probed on human metaphase chromosomes (Fig. 5). The results showed that MADS-IX is localized on chromosome10 at q21 region (close to centromere on the long arm).



Fig. 5. Localization of MADS-IX on human metaphase chromosomes. Centromere of chromosome 8 was used as a positive control probe. MADS-IX was found to localize around 10q21 chromosome region (close to centromere). Both the MADS-IX and CEP-8 are labeled with spectrum green (Vysis).

#### Identification of loss of MADS-IX on primary tumors by FISH:

Since our long term goal is to screen the MADS as FISH probes on primary tumor tissue sections to predict if that primary tumor is prone to developing metastasis or not, we made an attempt to screen MADS-IX as a FISH probe on normal tissue and primary tumor tissue sections of a patient who developed metastasis. We labeled chromosome 8 centromere (positive control) with spectrum orange and MADS-IX with spectrum green and screened normal tissue, primary tumor and positive lymph node tissue sections. Normal tissue section showed several red and green signals but the cell/nuclear morphology was not clear due to lot of fat around the cells. In the primary tumor tissue sections, as expected, the tumor cell nuclei were much larger than the normal cell nuclei and the FISH signals were clearer than that of the normal cell nuclei. Since we used 2 probes, we expected 3 patterns of labeling signals in the nuclei (presuming the centromere of chromosome 8 is not lost in these tumors), (i): 2 reds and 2 greens, if it is a normal cell nucleus or a tumor cell nucleus that did not lose MADS-IX; (ii): 2 reds and 1 green, if there is heterozygous loss of MADS-IX; (iii): 2 reds and no green, if there is homozygous loss of MADS-IX. As shown in figure 6, we observed clearly all the three patterns in the primary tumor section. Out of 50 cells with clear-cut morphology observed in the primary tumor section, 27 clearly were of first pattern (normal), 20 were of second pattern and 3 of third pattern. Similar FISH in the positive lymph node tumor section of the same primary tumor showed more pattern 3 cells rather than pattern 2 cells. Recently we screened another primary tumor that did not develop metastasis. Out of 50 cells observed, interestingly we did not record any cell that belonged to pattern 3 and only 3 cells that showed pattern 2 (loss of heterozygosity). These results are promising. As mentioned elsewhere in the present FISH preparations, we switched the dyes to avoid confusion between the spectrum green labeled target sequence signal and artifact green color signals generated by fat lobules in the primary tumor section.

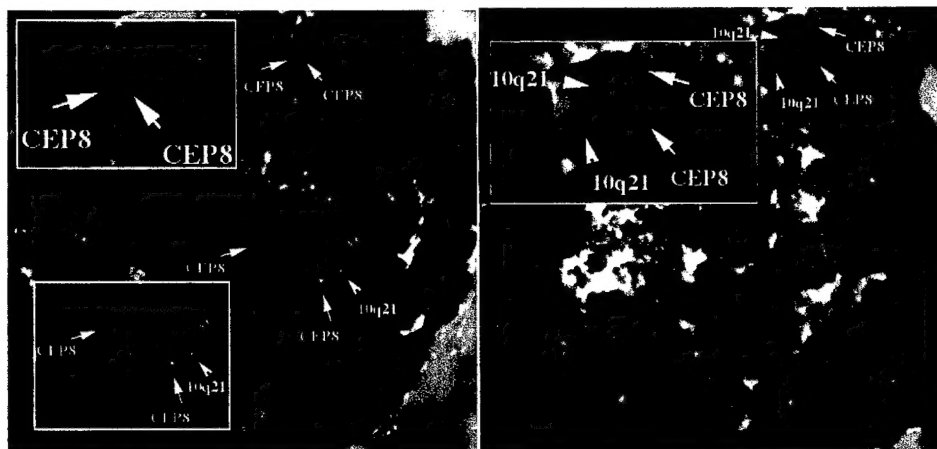


Fig. 6. Localization of MADS-IX in different cells of primary tumor tissue section. Chromosome 8 centromere (positive control with red signals) was labeled with spectrum orange and MADS-IX with spectrum green (green signals). 3 patterns of labeling signals in the nuclei were observed: (i): 2 reds and 2 greens, if it is a normal cell nucleus or a tumor cell nucleus (right side top inset) that did not lose MADS-IX; (ii): 2 reds and 1 green (left lower inset), if there is heterozygous loss of MADS-IX; (iii): 2 reds and no green, if there is homozygous loss of MADS-IX (left top inset).

#### Touch preparations:

Attempts are being made to use a simple method to prepare slides with tumor cells without using cut tissue sections from the tumor block. The tumor tissue material will be exposed by trimming of the paraffin material from the block and then by touching the tumor tissue side at several places on the slide which will allow some cells to attach from the block on to the slide surface. Conventional methods will be used to fix the cells followed by standard FISH procedure.

#### Expected FISH screening results:

Based on our results, we expect a panel of useful DNA markers which could differentiate primary tumors that did develop metastasis (Group-I) from those which did not develop metastasis (Group-II). We expect that a significant number of tumors in group-I will show loss of heterozygosity for majority of MADS and the corresponding positive lymph nodes should show homozygous loss. Similarly a significant number of tumors of group-II will show no loss for those MADS. However marker status comparisons will be made only between group-I primary tumors versus Group-II primary tumors. FISH results along with tumor data will be tabulated for analysis.

#### Evaluation of metastatic potential of BACS/MADS:

We identified 14 BACS that contain corresponding 14 MADS using gene bank search (Till to-date we could not find homology to MADS-IV in gene banks of NCBI and we do not have access to Celera database). We currently have obtained one BAC containing MADS-IX and the remaining will be purchased from Research Genetics (Huntsville, AL). The candidate BACs will be retrofitted with a selectable marker *neo* and then introduced individually into metastatic



human mammary MDA-MB-435 tumor cells following the procedure described by Mejia and Monaco (1997).

We have transfected a metastasis suppressor (nm23) gene and a metastasis promoter (ErbB2) gene with plxn vectors into MDA-MB-435 cells and successfully examined the primary tumors, lymph nodes and lungs in a SCID mouse model (Fig. 7). So we can easily transfect BAC/MADS into 435 cells. In this regard Dr. Athwal of Temple University, agreed to help us if we face any difficulty in the construction of retrofitted BAC/MADS.

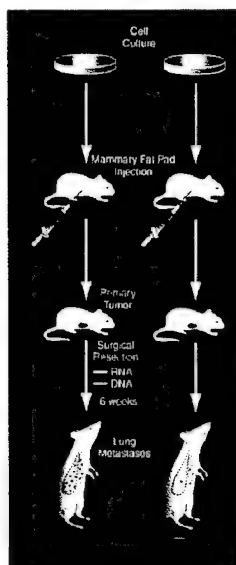


Fig. 7. *In Vivo* Spontaneous Metastasis Model: The left panel represents the mouse injected with control cells (vector only) and right panel with a retrofitted BAC/MADS which has the potential to inhibit or reduce metastasis (For details please see attached R-21 grant application).

## (6) Key Research Accomplishments:

- We isolated additional 4 candidate metastasis associated DNA sequences (MADS) using our "Total probe" method. Thus bringing the total number of MADS to 15 (11 MADS were isolated previously).
- Human multiple tissue Northern blot experiments reiterated the fact that MADS-IV (novel sequence having no homology in NCBI gene banks) is an expressed sequence and also revealed that it expresses in human breast and other tissue RNA samples.
- We have isolated three promising MADS (IV, IX and XI) that were found to be missing (partial/complete) in primary/metastatic cell DNA of patients other than the index cases.
- Screening of MADS-IX on different tumor samples and tumor cell lines (one of them had homozygous loss of PTEN gene) revealed that MADS-IX is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN region.
- Attempts were made to replace PCR method with Fluorescence in situ hybridization (FISH) for screening MADS on primary tumor tissue sections. Preliminary screening of

MADS-IX in the primary tumor (in a total of 50 cells) that had positive lymph nodes showed normal cells (27), loss of heterozygosity (20) and loss of homozygosity (3) while the primary tumor with negative lymph nodes showed normal cells (47), loss of heterozygosity (3) and loss of homozygosity (0) indicating the potential of the MADS as FISH probes in identifying the primary tumors that did and did not develop metastasis.

- The tumorigenesis cum metastasis SCID mouse model was used successfully by transfecting MDA-MB-435 breast carcinoma cells with a metastasis suppressor (nm23) and a metastasis promoter (ErbB2) to determine drug targeted genes associated with breast metastasis.

## **(7) Reportable outcomes:**

### **Manuscripts, abstracts and presentations**

- **Research manuscripts:**

#### **Published:**

R. Yuan, S Fan, P. Mohan R. Achary, D. M. Stewart, I. D. Goldberg, and E.M.R. Rosen, (2001) Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor (HGF/SF) in the setting of DNA damage. **Cancer Research** 61: 8022-8031 (copy attached; Annexure-I)

#### **Submitted:**

**P. Mohan R. Achary**, Zhao H, Fan Z, Herbst L, Mahadevia PS, Jones, J. Klinger HP, Vikram B. A candidate metastasis associated DNA marker for ductal mammary carcinoma. **Breast Cancer Research**. (copy attached; Annexure-II)

B. Mukherjee, H. Zhao, B. Parashar, B. M. Sood, P. S. Mahadevia, H. P. Klinger, B. Vikram, **P. Mohan R. Achary**. Microsatellite dinucleotide (T-G) repeat: A candidate marker for breast metastasis. **Cancer Detection and Prevention**. (copy attached; Annexure-III)

#### **In Preparation:**

C. Xue, H. Zhao, Jeffrey Segall and **P. Mohan R. Achary**. cDNA expression profile in EGFR and ErbB2 mediated metastasis in human breast carcinoma cell lines and their effect on spontaneous metastasis in a SCID mouse model. **Cancer Cell**.

Zhao H, Liu J, Datta PK, Klinger HP, Vikram B and **P. Mohan R. Achary**. 2002, Genes associated with nm23 mediated metastasis in breast carcinoma cells. **Tumor Biology**.

#### **Presentations:**



1. Zhao H, Fan Z, Mukherjee B, Herbst L, Jones J, Klinger HP, Vikram B and **P. Mohan R. Achary**, Characterization of Metastasis Associated Gene Sequences in Breast Carcinoma. Fifth Annual Einstein Postdoctoral Symposium, AECOM, December 4th, 2001 (Annexure-IV).
2. Invited talk on Metastasis associated genes in breast carcinoma by the faculty of the Department of Radiation Oncology, AECOM/MMC on January 7th, 2002.
3. Zhao H, Fan Z, Herbst L, Breining D, Jones JG, Mahadevia PS, Klinger HP, Vikram B, **P. Mohan R. Achary**. A candidate metastasis associated genetic marker for ductal mammary carcinoma. **93<sup>rd</sup> Annual AACR Meeting at San Francisco, California, Proceedings of American Association for Cancer Research**, April 6-10, 2002, (Annexure-V)
4. Selected as an external panelist in the **Human Genome Project conference** at Old Dominion University (Norfolk, VA) and presented a talk on breast metastasis, during June 14<sup>th</sup> and 15<sup>th</sup> 2002 (Annexure-VI)
5. Invited by the Department of Medical Oncology, Montefiore Medical Center to give a talk on the "Molecular markers in breast and cervical Cancer" on August 16<sup>th</sup> 2002.
6. Presentation (Molecular markers of metastasis in ductal mammary carcinoma) at the US Army 'Era of Hope' breast cancer research conference to be held during September 25<sup>th</sup> to 28<sup>th</sup>, 2002, in Orlando, Florida.

**Patents and licenses applied for and/or issued**

None

**Degrees obtained that are supported by this award**

None

**Development of cell lines, tissue or serum repositories (core facilities)**

- The PI procured Tissue Arrays (Glass slides consisting of 500 tumor sections from different cancers including breast cancer) from NCI. Attempts are currently being made to screen MADS as FISH probes on the tissue arrays.
- In our breast carcinoma tumorigenicity cum metastasis nude mouse model, we transfected human mammary metastatic cell lines (MDA-MB-435) with a metastasis suppressor (nm23) and a metastasis promoter (ErbB2) and generated cell lines from the primary tumors and metastatic lungs for cDNA microarray studies.

**Informatics such as databases and animal models etc.**

- The tumorigenicity cum metastasis mouse model is being currently used to study signal transduction pathways associated with nm23 and ErbB2 mediated breast metastasis.

- In collaboration with Dr. Roman Perez-Soler, we have used this mouse model to determine if a camptothecin drug called Db67 (which has been claimed to inhibit metastasis of certain tumors) will inhibit mammary carcinoma from becoming metastatic.

#### **Funding applied for based on work supported by this award**

- PI has resubmitted a R-21 grant application to NIH for the July 1<sup>st</sup>, 2002 deadline, based on the results obtained from the current US Army project (a copy of the application attached; Annexure-VII). In the first review (2001) it received a priority score of 236 which was just below the funding level. The reviewers of his application indicated strong approval for the novelty and importance of these molecular studies of mammary cancers but had some concerns as to the feasibility of some of the procedures to be used. In the interim, we have obtained additional results demonstrating the feasibility of these procedures and have resubmitted this application which is currently being reviewed.
- Dr. Richard A. Britten, Dept. of Radiation Oncology, East Virginia Medical Center, Norfolk, VA, as Principal Investigator and Dr. Achary as Co-PI has submitted an IDEA grant to US ARMY Ovarian Cancer Research Program on June 18<sup>th</sup> 2002.

#### **Employment or research opportunities applied for/or received on experiences/training supported by this award:**

- Dr. Herbst, Department of Pathology, AECOM, as Principal Investigator, and Dr. Achary as co-principal investigator, recently received a three-year grant from the Morris Animal Foundation.
- Dr. Achary has been selected as Editor for the journal, *Cytogenetic and Genome Research* (Annexure-VIII). He has been a reviewer for journals namely, *Gynecologic Oncology*, *American Journal of Pathology* and *Cancer Detection and Prevention*. Recently he has been selected as the 'Associate Administrator for Scientific Affairs' for the *International Cytogenetics and Genome Society*. He also has reviewed grant applications from the Foundation of Ohio Cancer Research Associates.

#### **(8) Conclusions:**

Fifteen candidate MADS have been isolated so far from 12 RDA experiments. Three of them (MADS-IV, IX and XI) were found to be promising markers for breast metastasis. RH mapping and homology search results indicated that MADS-IX is present close to PTEN gene on 10 q chromosomal arm. Screening of MADS-IX on different tumor samples and tumor cell lines (one of them with homozygous loss of PTEN gene) revealed that MADS-IX is neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN. Our attempts to supplement the PCR method with Fluorescence in situ hybridization (FISH) for screening MADS on primary tumor tissue sections were successful. Screening of MADS-IX as a FISH probe in 50 cells from a primary tumor that had positive lymph nodes, showed 27 normal cells 20 cells with loss of heterozygosity and 3 cells with loss of homozygosity while a primary tumor with negative lymph nodes showed 47 normal cells, 3 cells with loss of heterozygosity and

no cells with loss of homozygosity. These results indicate the potential of the MADS as FISH probes in distinguishing the primary tumors that did and that did not develop metastasis. The tumorigenesis cum metastasis SCID mouse model was used successfully by transfecting MDA-MB-435 breast carcinoma cells with a metastasis suppressor (nm23) and a metastasis promoter (ErbB2) to determine genes associated with signal transduction pathways leading to the identification of drug targets to control breast metastasis.

In spite of our sincere efforts, however, performing RDA experiments and characterizing MADS consumed most of our time. In addition, technical difficulties such as non-suitability of paraffin embedded archival tumor tissue samples for microdissection and of degraded DNA to use in RDA and PCR screening experiments, reduced the pace of our work. We therefore requested and obtained an extension of our project for a year from US Army BCRP to complete the ongoing experiments.

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# Altered Gene Expression Pattern in Cultured Human Breast Cancer Cells Treated with Hepatocyte Growth Factor/Scatter Factor in the Setting of DNA Damage<sup>1</sup>

Ren-qi Yuan, Saijun Fan, Mohan Achary, Donn M. Stewart, Itzhak D. Goldberg, and Eliot M. Rosen<sup>2</sup>

Department of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, New Hyde Park, New York 11040 [R.-q. Y., S. F., I. D. G., E. M. R.]; Departments of Radiation Oncology [M. A., E. M. R.] and Developmental and Molecular Biology [E. M. R.], Albert Einstein College of Medicine, Bronx, New York 10461; and Metabolism Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892 [D. M. S.]

## ABSTRACT

The cytokine hepatocyte growth factor/scatter factor (HGF/SF) protects epithelial and cancer cells against DNA-damaging agents via a pathway involving signaling from c-Met  $\rightarrow$  phosphatidylinositol-3-kinase  $\rightarrow$  c-Akt. However, the downstream alterations in gene expression resulting from this pathway have not been established. On the basis of cDNA microarray and semiquantitative RT-PCR assays, we found that MDA-MB-453 human breast cancer cells preincubated with HGF/SF and then exposed to Adriamycin (ADR), a DNA topoisomerase II inhibitor, exhibit an altered pattern of gene expression, as compared with cells treated with ADR only. [HGF/SF+ADR]-treated cells showed altered expression of genes involved in the DNA damage response, cell cycle regulation, signal transduction, metabolism, and development. Some of these alterations suggest mechanisms by which HGF/SF may exert its protective activity, e.g., up-regulation of polycystic kidney disease-1 (a survival-promoting component of cadherin-catenin complexes), down-regulation of 51C (an inositol polyphosphate-5-phosphatase), and down-regulation of TOPBP1 (a topoisomerase IIB binding protein). We showed that enforced expression of the cdc42-interacting protein CIP4, a cytoskeleton-associated protein for which expression was decreased in [HGF/SF+ADR]-treated cells, inhibited HGF/SF-mediated protection against ADR. The cDNA microarray approach may open up new avenues for investigation of the DNA damage response and its regulation by HGF/SF.

## INTRODUCTION

The cytokine HGF/SF<sup>3</sup> is a pleiotrophic mediator of multiple biological functions that plays significant roles in embryonic development, tissue and organ repair, tumorigenesis, and angiogenesis. HGF/SF has been found to protect various cell types against apoptosis induced by a variety of stimuli, including loss of contact with the substratum (1), exposure to staurosporine (a protein kinase inhibitor; Refs. 2, 3), and DNA damage (4-7). We have reported previously that various epithelial and carcinoma cell lines are protected by HGF/SF against apoptotic cell deaths induced by DNA-damaging agents, including ionizing radiation, ultraviolet (UV-C) radiation, and ADR (also known as doxorubicin; Ref. 5). ADR is a DNA intercalator and a DNA topoisomerase II $\alpha$  inhibitor that induces single- and double-strand DNA breaks similar to those induced by ionizing radiation.

Interestingly, preincubation with HGF/SF also reduced the number of residual DNA strand breaks at 24 h after exposure to ADR or ionizing radiation, suggesting that HGF/SF may also enhance the rate of DNA repair (i.e., strand rejoining; Ref. 6). The increased DNA

repair and the cell protection against DNA damage appeared to be attributable to at least in part, to: (a) activation of a cell survival pathway involving PI3K and c-Akt (protein kinase B); and (b) subsequent stabilization of the protein levels of the antiapoptotic mitochondrial pore-forming protein Bcl-X<sub>L</sub> (5, 6).

These studies have not revealed the downstream effector genes that mediate cytoprotection by HGF/SF. Cytoprotection by HGF/SF might involve nonnuclear events, such as inactivation of proapoptotic effectors (e.g., Bad and caspase-9) by c-Akt-mediated protein phosphorylation events (8, 9). However, it might also involve prolonged patterns of altered gene expression induced by HGF/SF in the DNA-damaged cells. The latter possibility was suggested by the observation that maximal protection required a preincubation of cells with HGF/SF for at least 48 h before exposure to ADR (5). Shorter preincubation periods yielded less protection, and application of HGF/SF only at the time of ADR treatment and during the 72-h postincubation period gave no protection.

To investigate the potential alterations of gene expression that might contribute to HGF/SF-mediated cell protection, we have used a cDNA microarray approach, using a previously studied model for HGF/SF protection (5). MDA-MB-453 human breast cancer cells were preincubated with HGF/SF, exposed to ADR, and then postincubated in ADR-free culture medium for 72 h to allow the repair processes to proceed. Alterations of mRNA expression were examined in cells treated with [HGF/SF+ADR], in comparison with cells treated with ADR alone.

## MATERIALS AND METHODS

**Sources of Reagents and Vectors and Sources of Reagents and Antibodies.** Recombinant human two-chain HGF/SF was generously provided by Dr. Ralph Schwall (Department of Endocrine Research, Genentech, Inc., South San Francisco, CA). ADR (doxorubicin hydrochloride) and MTT dye (thioazyl blue) were purchased from Sigma Chemical Co. (St. Louis, MO). Expression vectors encoding full-length and truncated or deleted forms of human CIP4 have been described earlier (10). These CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an NH<sub>2</sub>-terminal myc epitope tag.

**Cell Lines and Culture.** MDA-MB-453 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with FCS (5% v/v), nonessential amino acids (100 mM), L-glutamine (5 mM), streptomycin (100  $\mu$ g/ml), and penicillin (100 units/ml; all from BioWhittaker, Walkersville, MD). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

**ADR Treatment.** Subconfluent proliferating cells in 100-mm plastic dishes or 96-well plates were preincubated in the absence or presence of HGF/SF (100 ng/ml  $\times$  48 h) in serum-free DMEM and then sham-treated (control) or treated with ADR (10  $\mu$ M  $\times$  2 h, at 37°C) in complete culture medium (DMEM plus 5% FCS). Cultures were then washed three times to remove the ADR and postincubated in fresh drug-free complete culture medium at 37°C for 72 h (again in the absence or presence of HGF/SF, respectively). Cultures were then harvested for isolation of total cell RNA and cDNA microarray or semiquantitative RT-PCR analyses.

**Transient Transfections.** Subconfluent proliferating cells were transfected overnight using Lipofectamine (Life Technologies, Inc., Rockville, MD; 10  $\mu$ g of plasmid DNA/100-mm dish) and then washed to remove the excess vector

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<sup>2</sup>To whom requests for reprints should be addressed, at Department of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, 270-05 76th Avenue, New Hyde Park, NY 11040. Phone: (718) 470-7386; Fax: (718) 470-9756; E-mail: crosen@lij.edu.

<sup>3</sup>The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; ADR, Adriamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IP, immunoprecipitation; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR.

and Lipofectamine. As a control for transfection efficiency, cultures were cotransfected with 10  $\mu$ g of a  $\beta$ -galactosidase expression vector (pSV- $\beta$ -gal; Promega Corp., Madison, WI) under parallel conditions; and  $\beta$ -galactosidase was detected using a 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside staining kit (Gene Therapy Systems, Inc., San Diego, CA).

**MTT Cell Viability Assay.** This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt into an insoluble formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by spectrophotometry (11). To test the effect of CIP4 on HGF/SF-mediated cell protection, cells transiently transfected with CIP4 expression vectors (see above) were harvested using trypsin and seeded into 96-well dishes (2000 cell/well) in standard growth medium, incubated for 24–48 h to allow attachment and entry into the cell cycle, preincubated  $\pm$  HGF/SF (100 ng/ml  $\times$  48 h), treated with ADR (10 or 20  $\mu$ M  $\times$  2 h), postincubated for 72 h, and tested for MTT dye conversion. Cell viability was calculated as the amount of MTT dye conversion relative to sham-treated control cells. Ten replicate wells were tested for each experimental condition. Statistical comparisons were made using the two-tailed Student's *t* test.

**Isolation of RNA.** After cell treatments  $\pm$  ADR  $\pm$  HGF/SF, the total cellular RNA was extracted using TRIzol Reagent (Life Technologies, Inc.), according to the manufacturer's instructions. The RNA was treated with DNase and precipitated using 95% ethanol prior to cDNA synthesis. Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality of the RNA, and RNA concentrations were determined from absorbance measurements at 260 and 280 nm.

**cDNA Synthesis and Microarray Hybridization.** One hundred  $\mu$ g of total cellular RNA was annealed to oligo(dT) and reverse-transcribed in the presence of Cy3-labeled or of Cy5-labeled dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), using 10,000 units/ml of Superscript II reverse transcriptase (Life Technologies, Inc.). The resulting Cy3- and Cy5-labeled cDNAs were treated with RNase One (Promega) for 10 min at 37°C, combined, purified by using a Centricon-50 filtration spin column (Millipore, Bedford, MA), and concentrated to a final volume of 6.5  $\mu$ l. The cDNA was then combined with 12.5  $\mu$ l of hybridization solution and 1.0  $\mu$ l of blocking solution to a final volume of 20  $\mu$ l. The mixture was heated at 94°C for 2 min and centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a clean tube and incubated at 50°C for 1 h.

Hybridizations were performed on cDNA microarray glass slides prepared at the Albert Einstein College of Medicine microarray facility. Each slide contained 9216 unique human cDNA clones. The hybridization solution was placed on a pretreated microarray slide, covered with Hybri-slip, and then incubated in a hybridization chamber overnight at 50°C. After hybridization, the slide was washed at room temperature, first with 0.2  $\times$  SSC, 0.1% SDS for 20 min with gently shaking, and then with 0.2  $\times$  SSC two times (20 min each time). The slide was dried by spinning at low speed in a centrifuge for 5 min.

**Scanning, Griding, and Analysis.** The slides were scanned using a Microarray Scanner 4000A (Axon Instruments) at the Albert Einstein College of Medicine Cancer Center microarray facility. The scanner output images were localized by overlaying a grid on the fluorescent images, using the ScanAlyze software by Michael Eisen, Stanford University.<sup>4</sup> The fluorescent intensities were then calculated, using the program Copy of FUBAR! (the easy way out). The final reported intensity was the difference between average probe intensity and average local background intensity. Both final reported intensities (green and red) were filtered, and the spots with intensity  $<1.5$  were eliminated. The ratios of the red intensity to the green intensity and green intensity to red intensity for all targets were determined. The cDNA microarray results comparing cells treated with (HGF/SF+ADR) versus ADR alone are based on three completely independent experiments involving separate cell treatments, separate RNA isolations, and separate microarray assays. The microarray results comparing cells treated with HGF/SF alone vs 0 (control) are based on two completely independent experiments.

**Semiquantitative RT-PCR Analysis.** Aliquots of total cellular RNA (1.0  $\mu$ g) were subjected to first-strand cDNA synthesis using Superscript II reverse transcriptase (Life Technologies, Inc.), and the cDNA was diluted five times with water. One  $\mu$ l of the diluted cDNA was used for each PCR reaction. PCR amplifications were performed using a Perkin-Elmer DNA thermal cycler. The

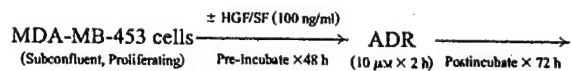
PCR primer sets used in this study are shown in Table 1. The PCR reaction conditions were individually optimized for each gene product studied. For each gene product, the cycle number was adjusted so that the reactions fell within the linear range of product amplification. PCR reaction conditions and cycle numbers are shown in Table 2. The  $\beta$ -actin and  $\beta_2$ -microglobulin genes were used as controls for loading. PCR products were analyzed by electrophoresis through 1.2% agarose gels containing 0.1 mg/ml of ethidium bromide, and the gels were photographed under ultraviolet illumination. The amplified cDNA product bands were quantitated by densitometry.

**IP and Western Blotting.** Subconfluent proliferating cells were harvested, and whole cell extracts were prepared, as described earlier (5). Each IP was carried out using 6  $\mu$ g of antibody and 1000  $\mu$ g of total extract protein. Precipitated proteins were collected using protein G beads, washed, eluted in boiling Laemmli sample buffer, and subjected to Western blotting. The c-Met IP antibody was c-Met COOH-terminal antibody SP260 (Santa Cruz Biotechnology, Santa Cruz, CA). The control IP antibody was an equivalent quantity (6  $\mu$ g) of normal mouse IgG (Santa Cruz Biotechnology).

Western blotting was performed as described earlier (5). The immunoprecipitated proteins or equal aliquots of total cell protein (50  $\mu$ g/lane) were electrophoresed, transferred, and blotted using the appropriate primary antibody. The primary antibodies were: (a) anti-c-Met antibody H-190 (sc-8307, rabbit polyclonal IgG; Santa Cruz Biotechnology; 1:500 dilution); (b) anti-phosphotyrosine antibody (Ab-4, mouse monoclonal; Calbiochem/Oncogene Research Products; 1:500 dilution); and (c) an anti-myc mouse monoclonal antibody (Invitrogen, Carlsbad, CA) at a 1:1500 dilution, to detect the myc epitope tagged wild-type and mutant CIP4 proteins.

## RESULTS

**cDNA Microarray Analyses.** The purpose of this study was to identify candidate genes, the expression of which is altered by HGF/SF in the setting of DNA damage, that might contribute to the HGF/SF-mediated protection against ADR. ADR is a DNA topoisomerase II $\alpha$  inhibitor that induces single- and double-stranded DNA breakage. The basic experimental protocol is described in "Materials and Methods" and is summarized in the diagram shown below:



cDNA microarray analysis:  
[HGF/SF + ADR] versus ADR

This design was chosen for several reasons. The main comparison was between [HGF/SF+ADR] versus ADR alone to identify genes for which expression was altered by HGF/SF during the response to DNA damage, because it is likely that some of these alterations may contribute to HGF/SF-mediated cell protection. However, a comparison of cells treated with HGF/SF versus CONTROL (sham treatment only) was also made. A postincubation period of *T* = 72 h after removal of ADR was used to examine well-established alterations in gene expression rather than transient changes occurring immediately after DNA damage. Furthermore, alterations in mRNA levels observed at *T* = 72 h are more likely to reflect changes in protein levels, because the mRNA alterations are of a prolonged duration.

Previous studies indicate that the ability of HGF/SF to protect cells against DNA-damaging agents is attributable to a c-Met receptor-mediated signaling pathway leading to the activation of a c-Akt-dependent survival pathway (6, 7). The HGF/SF-mediated cell protection was blocked by two fragments of the HGF/SF protein (designated NK1 and NK2) that bind strongly to the c-Met receptor, fail to fully activate c-Met signal transduction, and function as competitive antagonists of the full-length HGF/SF protein (5). Here, we show by IP-Western blotting that exposure of MDA-MB-453 cells to HGF/SF (100 ng/ml  $\times$  20 min) causes a large increase in the degree

<sup>4</sup> Internet address: eisen@genomic.stanford.edu, 1998–1999.



Table 1 Primers used for semiquantitative RT-PCR analyses

Gene name	Primer Sequences (5' → 3')	Genebank accession no.	Position in cDNA sequence	Expected size of product (bp)
<i>ATM</i> (ataxia telangiectasia mutated)	Sense: ctcatggttcagagtggtgagc Antisense: tacatgcgcgataagccaatcgc	NM_000051	8030-8757	728
<i>Polycystic kidney disease-1</i> (PKD-1)	Sense: ctctctatctgtgacagtcaccgcg Antisense: gtccagctgagagacgttggtgc	NM_000296	4528-5211	684
<i>Lysyl hydroxylase</i> (LH)	Sense: cgtcgatccctaattggccagcgc Antisense: aagatcgagctgtgcacagatgcc	L06419	2372-2986	615
<i>Lactate dehydrogenase type A</i> (LDH-A)	Sense: tagttctgccacctctgacgcacc Antisense: tataacacttggatagttggtgc	X02152.1	1330-1628	299
<i>U1 snRNP70</i>	Sense: cgcagatggcaagaagattgatgc Antisense: actccggctgcttgccttccgg	NM_003089	1700-2096	397
<i>Vascular endothelial growth factor</i> (VEGF)	Sense: atgtctatcagcgcagctactgc Antisense: caagctgcctgccttgcacgcg	XM_004512	150-548	399
<i>Phosphoglycerate kinase 1</i> (PGK-1)	Sense: ggtagtccttatgagccactagcc Antisense: cagccagcaggtatgccagaagcc	XM_010102	250-1011	762
<i>c-Myc</i>	Sense: cacatcagcacaactacgcagcgc Antisense: gactcagcagaaggttgagggttc	K02276	1331-1847	517
<i>Cdc42-interacting protein</i> (CIP-4)	Sense: caagacatggtgagcagcagc Antisense: gagatagtgccctcgtgcg	AJ000414	688-1550	863
<i>S100A9</i> (calgranulin B)	Sense: agggattcatcatgctgatggc Antisense: tggcctggcctctgattatgg	NM_002965	275-479	205
<i>TNF-inducible gene product B94</i>	Sense: gagtgcagtggcctggtcatgc Antisense: tctgactcagcactgcagagcc	M92357	3306-3944	639
<i>51C</i> (inositol-5'-polyphosphate phosphatase like-1, INPPL1) Primer Set #1	Sense: ctctctcgatttcagtgaggc Antisense: ccttcaatgctgcatcactcg	L36818	2062-2804	743
<i>51C</i> (inositol-5'-polyphosphate phosphatase like-1, INPPL1) Primer Set #2	Sense: tcagggcagttatctcttgc Antisense: acccaataatattaaggtgc	Y14385	4077-4522	446
<i>Topoisomerase binding protein-1</i> (TOPBP1)	Sense: cgacctagagtgacataatgc Antisense: gcttctcttaaaccttgc	NM_007027	4630-5123	494
<i>Protein Ser/Thr kinase</i> STK2	Sense: caactacagtgtaagcctgcgc Antisense: ctttaagggttaatacaatgcagg	NM_003157	2646-3144	499
<i>Protein tyrosine phosphatase</i> (PTPN2)	Sense: ctaagggaagacttatctctgc Antisense: tgtagcactgtgacttagtg	NM_002828	938-1359	422
<i>Human Gu protein</i>	Sense: acaggcagagctggaaggac Antisense: actgatgcggttaggtatc	U41387	1636-2123	488
<i>β-Actin</i>	Sense: tagcgggggtcaccacacgtgcccaccta Antisense: ctgaagcatttgcggtagcagtgaggag	XM_004814	541-1201	661
<i>β<sub>2</sub>-Microglobulin</i>	Sense: ctgcgcctctctctcttc Antisense: tgtcggatgatgaaccag	XM_007650	41-176	136

of activation (tyrosine phosphorylation) of c-Met (Fig. 1A). These findings support the role of the c-Met receptor in HGF/SF-mediated cell protection.

An illustration of cDNA microarrays comparing gene expression in cells treated with [HGF/SF+ADR] versus ADR alone and in cells treated with HGF/SF versus 0 (control) is provided in Fig. 1B. Gene products whose expression was consistently increased in [HGF/SF+ADR]-treated cells, relative to cells treated with ADR alone, by an average ratio of >1.7 in at least two of three completely independent experiments (i.e., separate cell treatments, RNA isolations, and microarray hybridizations) are listed in Table 3. Those gene products for which expression was consistently decreased in cells treated with [HGF/SF+ADR] relative to ADR alone (ratio <0.7 in at least two of three completely independent experiments) are listed in Table 4. The ratio values shown in these tables represent the mean  $\pm$  range ( $n = 2$ ) or mean  $\pm$  SD ( $n = 3$ ). Some of the cDNA sequences contained on the microarray slides corresponded to expressed sequence tags for which the full-length sequence is not available in public domain databases. Alterations in the expression of cDNAs corresponding to these cDNAs, for which there is little or no information available on the structure-function of the putative gene product, are not included in Tables 3 and 4.

Although the HGF/SF-induced alterations in gene expression in the setting of DNA damage were not usually very large (1.7–4.0-fold increases and 0.41–0.67-fold decreases), these changes were reproducible. Elevated mRNA levels in the [HGF/SF+ADR] group (relative to ADR alone) were observed for various different functional classes of genes, including genes involved in the DNA damage response (e.g., *ATM* and *FEN1*), cell cycle regulation (e.g., *Hs-cul-3* and *HsGAK*), signal transduction (e.g., *RHO B* and *CSBP1*), protein/

RNA synthesis and metabolism (e.g., *elF3*, *U1*, and *snRNP70*), development and cellular differentiation (e.g., *PKD1* and *IRX-2a*), general cellular metabolism (e.g., *LDH-A* and *PGK1*), and other functional categories (see Table 3). The abbreviations for these gene products are defined, and their functions (or putative functions) are shown in Table 3.

Genes for which the mRNA levels were reproducibly decreased in [HGF/SF+ADR]-treated cells (relative to ADR alone) included those in similar functional classes: including DNA damage response (e.g., *TOPBP1*), cell cycle regulation (e.g., *c-Myc* and *CIP-4*), signal trans-

Table 2 PCR reaction conditions for semiquantitative RT-PCR assays

Gene name	PCR cycle parameters	No. of cycles
<i>ATM</i>	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
<i>PKD-1</i>	94°C (1 min); 72°C (2 min)	28
<i>Lysyl hydroxylase</i>	94°C (1 min); 60°C (1 min); 72°C (1 min)	28
<i>LDH-A</i>	94°C (30 sec); 57°C (30 sec); 72°C (1 min)	25
<i>U1 snRNP70</i>	94°C (1 min); 60°C (1 min); 72°C (1 min)	30
<i>VEGF</i>	94°C (1 min); 57°C (1 min); 72°C (1 min)	28
<i>Phosphoglycerate kinase-1</i> (PGK-1)	94°C (1 min); 60°C (1 min); 72°C (1 min)	25
<i>c-Myc</i>	94°C (1 min); 57°C (1 min); 72°C (1 min)	25
<i>CIP-4</i>	94°C (1 min); 59°C (1 min); 72°C (1 min)	35
<i>S100A9</i>	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
<i>B94</i>	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
<i>51C</i> [INPPL1] Primer Set #1 (743-bp)	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
<i>51C</i> [INPPL1] Primer Set #2 (446-bp)	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	30
<i>TOPBP1</i>	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	30
<i>STK2</i>	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
<i>PTPN2</i>	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
<i>Human Gu protein</i>	94°C (30 sec); 55°C (30 sec); 72°C (1 min)	30
<i>β-Actin</i>	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	23
<i>β<sub>2</sub>-Microglobulin</i>	94°C (1 min); 54°C (1 min); 72°C (1 min)	28

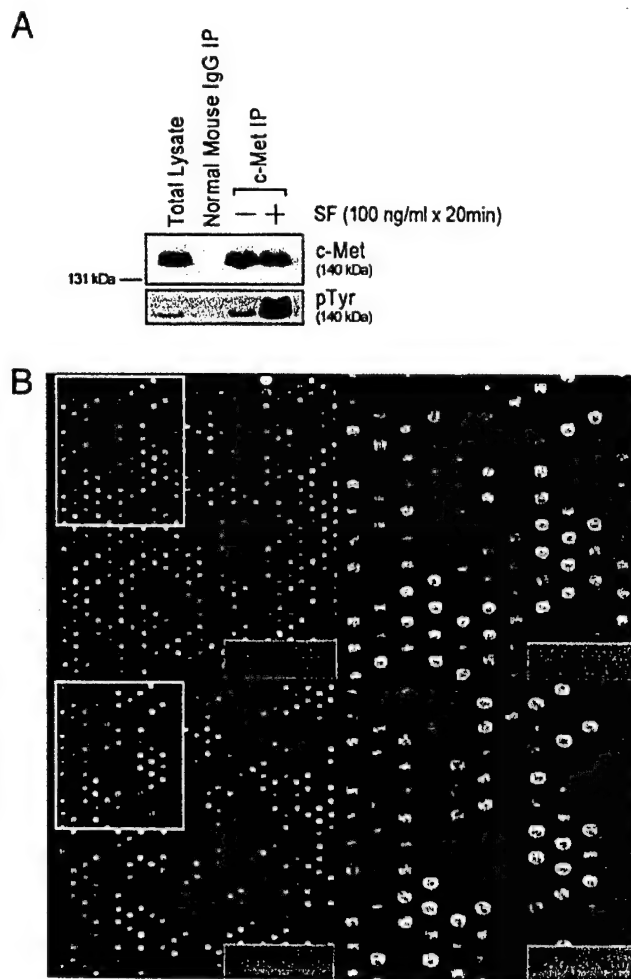


Fig. 1. HGF/SF activates c-Met and causes altered gene expression during DNA damage. **A**, HGF/SF causes activation (tyrosine phosphorylation) of the c-Met receptor. Subconfluent proliferating MDA-MB-453 cells were exposed to recombinant human HGF/SF (100 ng/ml  $\times$  20 min) and harvested for IP-Western blotting. Cells were immunoprecipitated using an anti-c-Met antibody or the same amount of normal IgG, as a negative control. IPs were then Western blotted using antibodies against c-Met and against phosphotyrosine residues. The basal level of phosphotyrosylated c-Met was low but was increased significantly by treatment with HGF/SF. No c-Met or phosphotyrosylated proteins were detected in the control (normal IgG) IP. **B**, illustration of cDNA microarray grids comparing gene expression in MDA-MB-453 cells treated with [(HGF/SF + ADR) versus ADR alone] (top panels) or with [(HGF/SF versus 0) (control)] (bottom panels). Cells were treated as described in the text. The panels on the right show magnified views corresponding to the boxed regions of the array on the left. cDNAs isolated from cells treated with [(HGF/SF + ADR) or HGF/SF] were labeled with Cy5 (red dye), whereas cDNAs from cells treated with ADR alone or 0 were labeled with Cy3 (green dye). Spots showing red (or green) fluorescence correspond to genes overexpressed (underexpressed) in cells treated with [(HGF/SF + ADR) relative to ADR alone and with HGF/SF relative to 0]. Yellow spots correspond to genes equally expressed under the conditions being compared, whereas the absence of fluorescence indicates genes under either experimental condition. Note that alterations in gene expression, indicated by red or green spots, are more prominent in the comparison of [(HGF/SF + ADR) versus ADR] than [(HGF/SF versus 0)].

duction (e.g., *51C* and *STK2*), and protein and RNA metabolism (e.g., *human Gu protein*). Few or no gene products for which expression was reduced were observed in several functional classes, including development and differentiation, transcriptional regulation, and general cellular metabolism. However, in interpreting the significance of the lack of genes whose expression was decreased in certain functional classes, it should be noted that: (a) the number of genes included in each functional class is influenced by the ratio cutoffs, which is arbitrary; (b) fewer genes showed decreased than increased expression, based on the ratio criteria chosen; and (c) the inclusion of

genes in the different functional categories was somewhat arbitrary, because some genes could be included in more than one category.

Table 5 shows a cDNA microarray comparison of gene expression in MDA-MB-453 cells treated with HGF/SF relative to untreated control cells. These data indicate that the number of genes whose expression is reproducibly altered and the magnitude of the alterations are relatively small when the experiment is performed in the absence of treatment with ADR. However, it was noted that *51C* (*INPPL1*), which was decreased in [(HGF/SF + ADR)-treated cells relative to ADR alone], was also decreased in HGF/SF-treated cells relative to control.

**RT-PCR Assays.** Because false-positive results are commonly observed in cDNA microarray analyses, we sought to confirm some of the gene expression alterations shown in Tables 3 and 4, via semi-quantitative RT-PCR assays, using techniques described before by us (12, 13). The PCR primers and reaction conditions are provided in Tables 1 and 2, respectively. For each PCR assay, the reaction conditions and cycle numbers were individually optimized and adjusted so that the reaction fell within the linear range of product amplification.  $\beta$ -Actin and  $\beta_2$ -microglobulin, two genes whose expression was not altered, were used as controls for loading. The levels of amplified PCR products were quantitated by densitometry and expressed relative to  $\beta$ -actin. Figs. 2 and 3 show semi-quantitative RT-PCR results for genes whose expression was either increased (Fig. 2) or decreased (Fig. 3) in cells treated with [(HGF/SF + ADR)] relative to ADR alone.

In general, qualitative agreement between the cDNA microarray and RT-PCR results was quite good, although there were differences in the quantitative extent of the gene expression alterations between the two assay methodologies. Figs. 2 and 3 show 16 different genes for which expression was either increased ( $n = 7$ ) or decreased ( $n = 9$ ) in [(HGF/SF + ADR)-treated cells] by both cDNA microarray and semi-quantitative RT-PCR analyses. Genes confirmed to be increased in the [(HGF/SF + ADR)] group included: *ATM* (ataxia-telangiectasia mutated), *PKD1* (polycystic kidney disease-1), *lysyl hydroxylase*, *LDH-A* (lactate dehydrogenase-A), *U1 snRNP70* (U1 small nuclear ribonucleoprotein,  $M_r$  70,000), *VEGF* (vascular endothelial growth factor), and *PGK1* (phosphoglycerate kinase). Genes confirmed to be decreased in the [(HGF/SF + ADR)] group included: *c-Myc*, *CIP4* (*cdc42-interacting protein-4*), *S100A9* (calgranulin), *B94* (a TNF-inducible gene product), *51C* (an inositol polyphosphate-5-phosphatase, also known as *INPPL1* and *SHIP-2*), *TOPBP1* (a DNA topoisomerase IIB binding protein), *STK2* (a serine/threonine protein kinase), *PTPN2* (a protein tyrosine phosphatase), and *Gu protein* (an RNA helicase).

Some of these alterations, although novel and not otherwise predictable, make sense within the context of explaining how HGF/SF may protect DNA-damaged cells, as will be considered in depth in the "Discussion." The down-regulation of *51C* in [(HGF/SF + ADR)-treated cells] was of particular interest because: (a) a decrease in *51C* mRNA levels was also noted in cells treated with HGF/SF alone (related to sham-treated control cells); and (b) *51C* is a lipid phosphatase, analogous to *PTEN*, except that *51C* removes the 5-phosphate whereas *PTEN* removes the 3-phosphate (14). Thus, *51C*, similar to *PTEN* (15), might be expected to inhibit c-Akt activation (see "Discussion"). Thus, we also examined *51C* expression levels by semi-quantitative RT-PCR using a completely different set of primers. Similar results were obtained for *51C* using both sets of PCR primers (see Fig. 3).

Finally, it is noted that the RT-PCR assays provide additional information not obtained in the microarray comparisons. The RT-PCR assays allow comparisons of gene expression in cells treated with ADR, relative to control, a comparison not made by cDNA microarray

Table 3 Genes whose expression is increased in [HGF/SF+ADR]-treated cells relative to ADR alone

Gene name	Function	Ratio
<b>DNA damage response</b>		
ATM (ataxia-telangiectasia mutated)	DNA damage signaling, nuclear PI-3-kinase domain protein	2.9 ± 0.0
FEN1	Flap endonuclease-1, implicated in base excision repair pathway	2.1 ± 0.04
<b>Cell cycle regulation</b>		
CENP-F kinetochore	Micronubule motor protein, component of centromere	2.7 ± 0.3
Hs-cul-3	Homology to cullin/cdc53 family, ? role in cell proliferation control	2.4 ± 0.15
HsGAK	Uniquitously expressed perinuclear cyclin G-associated kinase	2.3 ± 0.4
NuMa gene (clone T33)	Nuclear mitotic protein, mitotic centromere function	2.1 ± 0.04
Cell growth regulator CGR19	Ring finger gene induced by p53	2.0 ± 0.2
Cyclin G2	May mediate proteolysis of G1 family cyclins	1.9 ± 0.2
<b>Signal transduction-related</b>		
RHO B transforming protein	Endosomal Rho protein, roles in receptor trafficking and apoptosis	4.0 ± 1.7
Dual specificity tyr phosphorylat. regulated kinase	Homolog of Drosophila kinase midbrain, ? role in brain development	2.1 ± 0.2
CSAids binding protein-1 [CSBP1]	Also known as p38, homolog of yeast Hog1 MAPK, stress response signaling	2.1 ± 0.15
Protein phosphatase PPP2R2A [PR53]	M <sub>r</sub> 53,000 regulatory subunit of Ser/Thr protein phosphatase 2A	1.9 ± 0.2
RAB5A	ras-related small GTPase, regulator of vesicle trafficking	1.6 ± 0.1
<b>Protein and RNA metabolism</b>		
Lysyl hydroxylase [LHI, also known as PLOD]	Collagen modification, defective in Ehlers-Danlos syndrome VI	2.4 ± 0.7
eIF3	Eukaryotic translation initiation factor	2.2 ± 0.3
U1 snRNP70 (small nuclear ribonucleoprotein)	Associated with RNA processing and ubiquitination	2.1 ± 0.3
SAP49	Spliceosomal associated protein, RNA processing	2.1 ± 0.5
Cellular and nucleic acid binding protein		2.0 ± 0.3
SNC19	Putative novel human serine protease mapping to chr. 11q24-25	1.9 ± 0.2
β-COP	Golgi transport protein, component of COT1 complex	1.8 ± 0.02
<b>Cytokine and cytokine-induced</b>		
Vascular endothelial growth factor (VEGF)	Stimulates endothelial cell proliferation and angiogenesis	2.8 ± 0.9
VEGF-related protein [VRP]	FLT4 ligand, VEGF family protein	2.1 ± 0.4
Interferon-induced M <sub>r</sub> 17,000 protein	Precursor of 15 kDa protein homologous to ubiquitin	1.8 ± 0.2
<b>Development and differentiation</b>		
Keratin 17	Soft epithelial keratin 9 (e.g., hair follicle)	3.8 ± 1.5
B4-2 protein	Proline-rich natural killer cell protein	2.6 ± 0.8
Keratin 19	Intermediate filament protein	2.5 ± 0.7
Iroquois class homeodomain protein IRX-2a	Transcription factor involved in embryonic patterning, regionalization	2.3 ± 0.4
Polycystic kidney disease-1 [PKD1]	Component of cadherin-catenin complex, endothelial survival	2.0 ± 0.4
Cancellous bone osteoblast	mRNA expressed in osteoblasts, function unknown	2.0 ± 0.1
SM22a homologue [TAGLN2]	Marker of differentiated smooth muscle (SM)-like cells	1.7 ± 0.2
<b>Transcriptional regulation</b>		
RIP140	Nuclear receptor-interacting protein, transcriptional coactivator	2.1 ± 0.1
hlf-1	Novel zinc finger protein isolated from a brain cDNA library	2.1 ± 0.0
DGS-1	DiGeorge (velocardiofacial) syndrome candidate gene	2.0 ± 0.45
<b>General cellular metabolism</b>		
Lactate dehydrogenase-A [LDH-A]	Enzyme involved in anaerobic glycolysis	4.1 ± 1.0
Phosphoglycerate kinase [PGK1]	Glycolytic enzyme, induced by hypoxia-inducible factor HIF-1	4.0 ± 1.4
Hexokinase-1	Early glucose metabolic enzyme	2.2 ± 0.3
Glucosylceramidase precursor	Degradation of GlcCer, mutated in Gaucher's disease	2.0 ± 0.4
Phosphoglycerate mutase 1 [PGAM1]	Late glycolytic pathway enzyme	1.7 ± 0.25
<b>Cytoskeletal and structural proteins</b>		
Ezrin-radixin-moesin phosphoprotein 50 [EBP50]	PDZ phosphoprotein, linkage of cell membrane to cytoskeleton	3.8 ± 1.0
p16-Arc [ARC16]	Arp 2/3 complex subunit, control of actin polymerization	1.8 ± 0.1
<b>Miscellaneous and unknown function</b>		
XAP-5	Unknown function	3.1 ± 0.7
OriP binding protein [OBP1]	Binds to Epstein Barr virus replication origin	2.1 ± 0.2
JTV-1	Gene overlapping PMS2, function unknown	1.9 ± 0.2
MAC30 (3' end)	Meningioma expressed protein	1.9 ± 0.2
Sm-like (CuSm)	Cancer-associated Sm motif-like domain protein	1.7 ± 0.2

analysis. Thus, in Fig. 2, it was observed that in most cases, the main effect of HGF/SF was not to alter gene expression by itself but to block the ADR-induced reduction of mRNA levels that were observed in the absence of HGF/SF. In Fig. 3, with the exception of *51C* and *PTPN2*, HGF/SF by itself did not significantly alter gene expression; but its main effect was to block the ADR-induced up-regulation of mRNA levels. However in some cases, the mRNA levels in [HGF/SF+ADR]-treated cells were reduced to below control levels (e.g., *CIP4* and *TOPBP1*).

**Role of CIP4 in HGF/SF-mediated Protection against ADR.** The cdc42-interacting protein-4 (CIP4) was originally identified as a protein that binds to the activated form of cdc42, a Rho-like small GTPase, and was subsequently found to bind to the Wiskott-Aldrich syndrome protein (WASP) through its COOH terminus and to microtubules through its NH<sub>2</sub> terminus (Refs. 10, 16; illustrated in Fig. 4A). Although CIP4 is not known to be involved in cell survival or apoptosis pathways, the finding that CIP4 mRNA expression is up-regulated by ADR and that HGF/SF blocks the ADR-induced up-

regulation of CIP4 raises this possibility. To determine whether CIP4 could modulate the survival of MDA-MB-453 cells in response to of ADR or HGF/SF, MDA-MB-453 cells were transfected with expression vectors encoding wild-type (wt) or mutant (truncated or deleted) forms of CIP4 containing an NH<sub>2</sub>-terminal myc epitope tag and then assayed for their survival response. The MTT assay, which measures cytotoxicity as the loss of mitochondrial function (i.e., the ability to reduce a tetrazolium dye to formazan) was used to quantitate cell viability (11). Expression of these proteins was confirmed by Western blotting of transfected cells using an anti-myc antibody (see Fig. 4B).

Cells transfected with wild-type CIP4 (wtCIP4) showed an increased sensitivity to ADR, as well as a significantly decreased degree of cytoprotection by HGF/SF (Fig. 4C), consistent with a role as a modulator of DNA damage or apoptosis response pathways. In the absence of HGF/SF, the decrease in cell survival (viability) in wt-CIP4-transfected cells (relative to the empty vector transfected control) treated with ADR alone was greater at 10 μM ADR (-28%;  $P < 0.001$ , two-tailed *t* test) than at 20 μM ADR (-10%;  $P < 0.05$ ,



Table 4 Genes whose expression is decreased in [HGF/SF+ADR]-treated cells, relative to ADR alone

Gene name	Function	Ratio
<b>DNA damage response</b>		
<i>P</i> glycoprotein 3/MDR3 [PGY3]	Homologue of multidrug resistance protein MDR-1, drug transport	0.51 ± 0.07
Topoisomerase binding protein-1 [TOPBP1]	BRCT domain protein, binds DNA topoisomerase IIB	0.61 ± 0.05
<b>Cell cycle regulation</b>		
<i>c-Myc</i>	Proto-oncogene, functions in growth, differentiation, apoptosis	0.41 ± 0.04
CIP4 ( <i>cdc42</i> -interacting protein)	Interacts with Wiskott-Aldrich protein, localized in cytoskeleton	0.42 ± 0.08
<i>ras</i> inhibitor (3' end)	Effector or regulator of H-Ras activity	0.60 ± 0.07
<b>Signal transduction-related</b>		
<i>M</i> , 180,000 transmembrane PLA2 receptor	Receptor for secretory phospholipases A2, internalizes PLA2	0.51 ± 0.05
Protein tyrosine phosphatase PTPN2	Also known as PT PTP (T cell protein tyrosine phosphatase)	0.57 ± 0.17
Proto-oncogene <i>c-mer</i> [MERTK]	Member of Axl subfamily of receptor tyrosine kinases	0.57 ± 0.10
Protein serine/threonine kinase STK2	Homologue of cell cycle regulatory kinase NIMA	0.60 ± 0.05
S1C [INPPL1]	Inositol polyphosphate-5'-phosphatase-like (also known as SHIP-2)	0.61 ± 0.10
<b>Apoptosis-related</b>		
CD40L receptor	Receptor for CD154, member of TNF death receptor family	0.57 ± 0.08
<b>Protein and RNA metabolism</b>		
Human Gu protein	RNA helicase, member of DEXD box family, target of adriamycin	0.46 ± 0.01
Cathepsin K precursor	Lysosomal acid cysteine protease, mediates proteolysis of bone	0.49 ± 0.10
<b>Cytokine and cytokine-induced</b>		
B94	TNF-induced gene product, unknown function	0.56 ± 0.07
Tazarotene-induced gene 2 [TIG2]	Novel retinoid-responsive gene, deficient in psoriatic skin	0.57 ± 0.02
IGF-1 (somatomedin-C)	Insulin-like growth factor-1	0.58 ± 0.02
FGF-7 (fibroblast growth factor-7)	Also known as keratinocyte growth factor, epithelial-specific growth factor	0.59 ± 0.07
<b>Development and differentiation</b>		
None		
<b>Transcriptional regulation</b>		
None		
<b>General cellular metabolism</b>		
None		
<b>Cytoskeletal and structural proteins</b>		
S100A9 (calgranulin B)	Secretory protein, ? roles in inflammation, eicosanoid metabolism	0.42 ± 0.25
Human triadin	Integral membrane protein, binds calsequestrin	0.58 ± 0.16
Vascular cell adhesion molecule VCAM1	Ig superfamily, interacts with $\alpha$ -4 integrins, cell trafficking	0.58 ± 0.07
Ankyrin G	Axon nodal protein involved in assembly of specialized structures	0.59 ± 0.13
<b>Miscellaneous and unknown function</b>		
hORC2L (origin recognition complex)	Putative replication initiation protein	0.58 ± 0.05
CHD2	Chromodomain helicase DNA-binding protein 2	0.60 ± 0.13
Rip-1 (Rev-interacting protein)	Interacts with HIV Rev protein, ? function	0.67 ± 0.01

two-tailed *t* test). This finding might reflect a greater degree of up-regulation of endogenous CIP4 expression at the higher dose of ADR, so that the transfected wtCIP4 has a smaller effect. For cells treated with HGF/SF, at both 10 and 20  $\mu$ M ADR, the survival of the wtCIP4-transfected cells was significantly lower than the empty vector-transfected cells ( $P < 0.001$ ).

The quantitative degrees of cell protection by HGF/SF were calculated based on the following equation, where  $(S/S_0)$  = cell viability relative to control:

$$\text{Protection by HGF/SF (\%)} = \left[ \frac{(S/S_0)_{\text{HGF/SF+ADR}}}{(S/S_0)_{\text{HGF/SF, 0ADR}}} - \frac{(S/S_0)_{\text{HGF/SF+ADR}}}{(S/S_0)_{\text{HGF/SF+ADR}}} \right] \times 100$$

The % protection values at doses of 10 and 20  $\mu$ M ADR were averaged and plotted in the bottom panel of Fig. 4C. On the basis of these calculations, transfection of wtCIP4 reduced the HGF/SF-

mediated cell protection from ~85 to 40%. On the other hand, there was no effect of wtCIP4 on cell viability in the absence of ADR (100% of control).

Expression vectors encoding mutant forms of CIP4 included a deletion of the microtubule binding domain (CIP4 118–545), a deletion missing the *cdc42* binding region (CIP4  $\Delta$  383–481) and a deletion of the COOH-terminal WASP binding domain (Fig. 4A). In general, these deletion mutants had little or no effect on the degree of HGF/SF-mediated cell protection, nor did they affect cell viability in the absence of ADR (Fig. 4C). However, cells transfected with the mutant CIP4 cDNAs did show an increase in cell viability (by ~15–20%) at 20  $\mu$ M ADR in the absence of HGF/SF. This finding may be attributable to their function as dominant inhibitors of the endogenous wild-type CIP4, although that conclusion cannot be made from this experiment alone.

Similar findings were obtained using another cell type that is also

Table 5 Genes whose expression is altered in HGF/SF-treated cells, relative to untreated control cells

Gene name	Function	Ratio
<b>Gene products increased in HGF/SF-treated cells</b>		
Interleukin-8 (IL-8)	Proinflammatory & angiogenic cytokine, neutrophil chemotaxis	1.6 ± 0.01
(Clone ch13 lambda 7) $\alpha$ -tubulin	Microtubule protein	1.6 ± 0.02
Cytochrome c oxidase VIIc subunit [COX7C]	Subunit of COX holoenzyme, mitochondrial energy production	1.5 ± 0.01
Tubulin $\beta$ -1 chain	Microtubule protein	1.5 ± 0.01
<b>Gene products decreased in HGF/SF-treated cells</b>		
S1C [INPPL1]	Inositol polyphosphate-5'-phosphatase-like (aka. SHIP-2)	0.43 ± 0.0
il-TMP (intestine/liver tetraspan protein)	Integral membrane protein, density-dependent growth regulation	0.57 ± 0.13
Integrin $\alpha$ -8 subunit, 3' end	Integrin expressed in developing brain and mesangial cells	0.58 ± 0.02
Topoisomerase IIB [TOP2B]	Nuclear enzyme involved in DNA replication and transcription	0.65 ± 0.01
Corticotrophin releasing factor receptor precursor	Mediates release of corticotrophin (ACTH)	0.69 ± 0.01
Osteoblast mRNA for osteonidogen	Basement membrane component, entactin/nidogen family	0.69 ± 0.05
Janus kinase 1 [JAK1]	Mediates tyrosine phosphorylation of STAT1	0.70 ± 0.01
MutS homologue 3 [MSH3]	DNA mismatch repair enzyme	0.72 ± 0.01

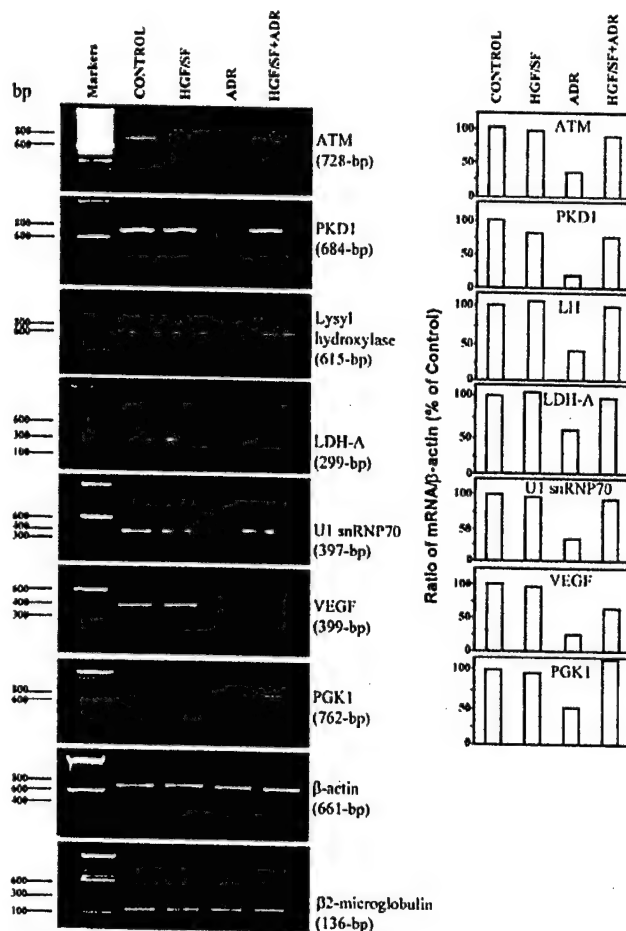


Fig. 2. Semiquantitative RT-PCR analyses of genes for which expression was increased in MDA-MB-453 cells treated with [HGF/SF+ADR] relative to ADR alone. Subconfluent proliferating cells were preincubated  $\pm$  HGF/SF (100 ng/ml  $\times$  48 h), treated  $\pm$  ADR (10  $\mu$ M  $\times$  2 h), washed three times to remove the ADR, and postincubated for 72 h in fresh drug-free medium, as described in the text. RNA was collected, and RT-PCR assays were performed (see "Materials and Methods" and Tables 1 and 2 for methodological details).  $\beta$ -Actin and  $\beta_2$ -microglobulin were used as controls for loading. The amplified PCR products were quantitated by densitometry and expressed relative to  $\beta$ -actin, as a percentage of the control (0 HGF/SF, 0 ADR).

protected against ADR-induced DNA damage by preincubation with HGF/SF, DU-145 human prostate cancer cells (6). Thus, wtCIP4, but not the mutant or truncated forms of CIP4, blocked the HGF/SF-mediated protection against ADR (data not shown). These findings are consistent with a role for CIP4 as a regulator or modulator of cell survival in the setting of DNA damage.

## DISCUSSION

These studies revealed an interesting pattern of up-regulation and down-regulation of genes in MDA-MB-453 cells treated with [HGF/SF+ADR], as compared with ADR alone. Admittedly, some of these gene products may be altered simply because of the higher proportion of surviving cells in the [HGF/SF+ADR]-treated group relative to the ADR-treated group. Gene products of this type might include lactate dehydrogenase [LDH-A] and phosphoglycerate kinase [PGK1], which were increased in [HGF/SF+ADR]-treated cells. However, the complexity of the findings, including many genes that were either increased or decreased in ADR-treated cells, suggest a more selective pattern of altered gene regulation.

We have reported previously that in addition to protecting cells

against cytotoxicity and apoptosis induced by DNA damage, HGF/SF enhanced the ability of carcinoma cells, including MDA-MB-453 cells, to repair DNA strand breaks induced by ADR or X-rays (6). The observation that cells treated with [HGF/SF+ADR] show altered expression of certain gene products involved in DNA damage response pathways is consistent with that prior finding. For example, *ATM* (ataxia-telangiectasia mutated), a nuclear protein kinase involved in DNA damage signaling (17), and *FEN1* (flap endonuclease-1), an enzyme implicated in the base excision repair pathway (18), were up-regulated in [HGF/SF+ADR]-treated cells. A mutation or deletion of the *ATM* gene leads to a defect in the repair of double-strand DNA breaks and increased sensitivity to ionizing radiation.

We also found that ADR caused the down-regulation of the *PKD1* (polycystic kidney disease-1) gene product, and HGF/SF blocked the ADR-induced down-regulation of *PKD1* expression. *PKD1* has been identified as a developmentally regulated gene, the absence of which is linked to type I autosomal dominant polycystic kidney disease (19). The function of this gene is not well understood, but *PKD1* was found to encode a large cell membrane protein associated with the cadherin-catenin cell:cell adhesion complex (20). Interestingly, the *PKD1* gene product was shown recently to play roles in maintaining the structural integrity of blood vessels (21) and in protecting MDCK epithelial cells against apoptosis (22). We had reported previously that HGF/SF

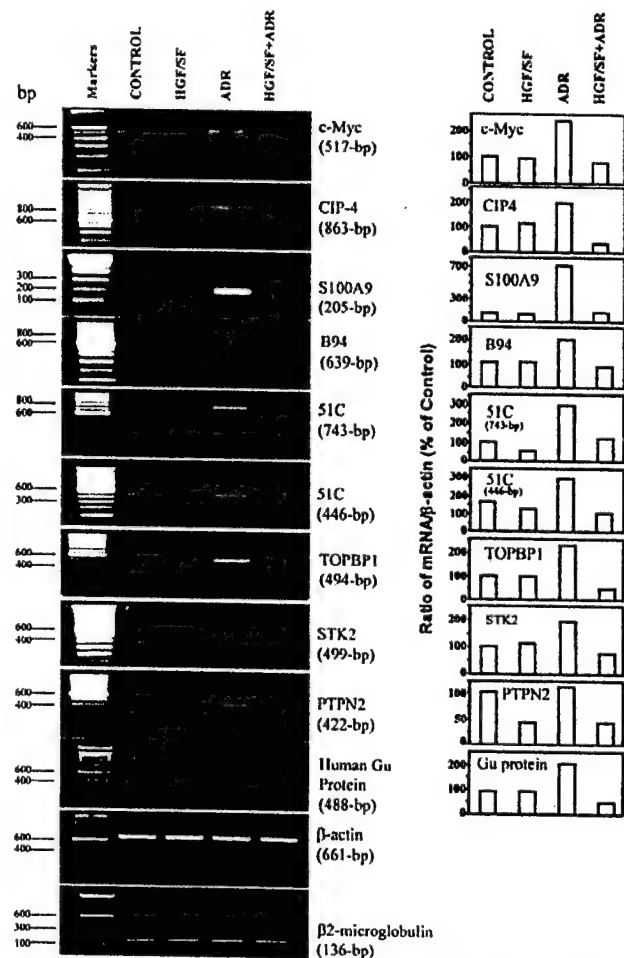


Fig. 3. Semiquantitative RT-PCR analyses of genes for which expression was decreased in MDA-MB-453 cells treated with [HGF/SF+ADR] relative to ADR alone. Assays were performed as described in the Fig. 2 legend. Note that 51C was analyzed using two completely different sets of PCR primers.

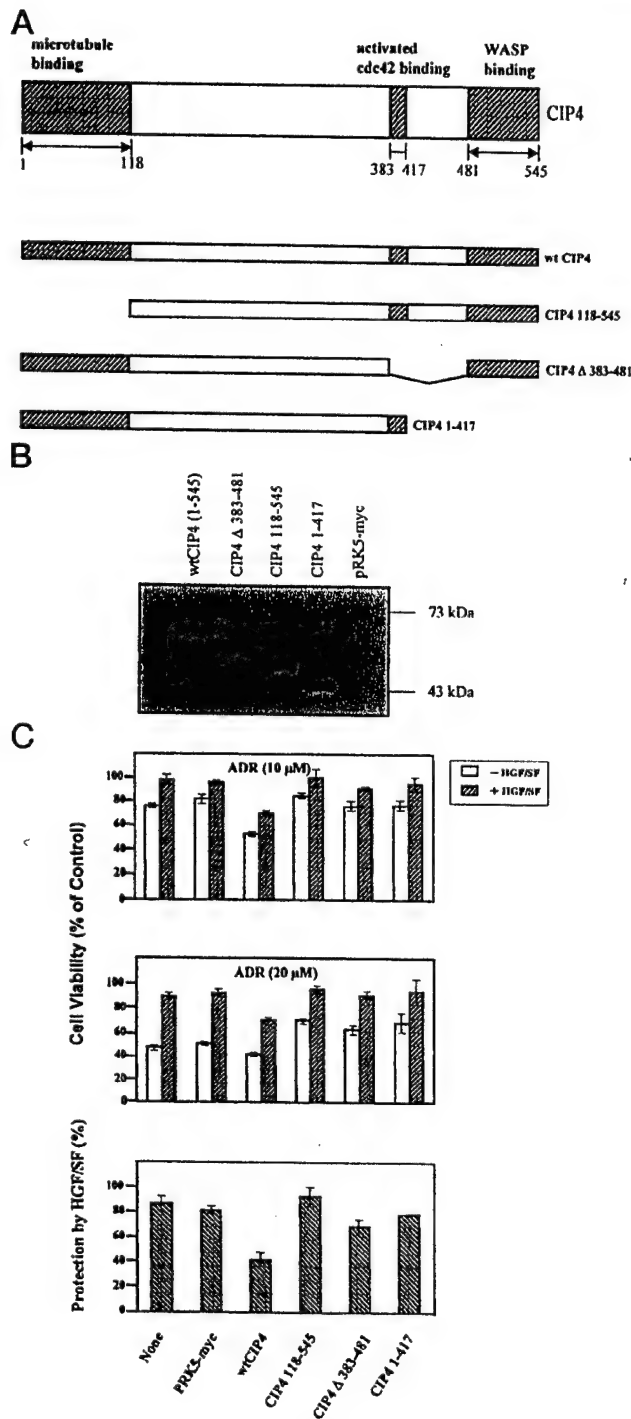


Fig. 4. Effect of genetic manipulation of cdc42-interacting protein (CIP4) expression on HGF/SF-mediated protection of MDA-MB-453 cells. **A**, schematic diagrams of CIP4 expression vectors. The human CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an NH<sub>2</sub>-terminal myc epitope tag. **B**, expression of wild-type and mutant CIP4 proteins. Cells were transfected with the different CIP4 expression vectors as described below (**C**), and the dishes were incubated for 24 h to allow expression of the encoded proteins. Proteins of the expected sizes were detected by Western blotting, using an antibody against the myc epitope tag. Cells transfected with the empty pRK5-myc vector showed no myc-tagged proteins. **C**, effect of transient expression of wild-type (wt) and mutant CIP4 proteins on HGF/SF-mediated cell protection. Subconfluent proliferating cells in 100-mm dishes were transiently transfected overnight with 10  $\mu$ g of each vector, in the presence of Lipofectamine. Cells were washed, subcultured into 96-well dishes, pre-incubated  $\pm$  HGF/SF (100 ng/ml  $\times$  48 h), exposed to ADR (10 or 20  $\mu$ M  $\times$  2 h), washed, postincubated for 72 h in fresh drug-free medium, and assayed for MTT dye conversion. Cell viability values (means  $\pm$  SE) are based on 10 replicate wells. For each experimental condition, cells treated with [HGF/SF + ADR] showed higher

protects both vascular endothelial and MDCK epithelial cells against DNA damage-induced apoptosis (4, 5). Thus, inhibition of the down-regulation of *PKD1* by HGF/SF may be a cytoprotective function, one which merits further investigation.

On the other hand, the expression of the topoisomerase binding protein TOPBP1, which binds DNA topoisomerase IIB and also shows DNA strand break binding activity (23-25), was decreased in cells treated with [HGF/SF + ADR]. ADR causes DNA strand breakage in part by converting the DNA topology enzyme topoisomerase II into a DNA cleaving enzyme (26). It is thought that topoisomerase binding proteins such as TOPBP1 may contribute to or potentiate ADR-mediated DNA damage, but the role of TOPBP1 in this process remains to be established. The finding that ADR up-regulates TOPBP1 expression and that the up-regulation is blocked by HGF/SF is provocative, because it suggests a potential mechanism by which HGF/SF might modulate the DNA damage and repair process, upstream of DNA-damage induced apoptosis. HGF/SF blocked the ADR-induced up-regulation of the human Gu protein. Gu is a DEXD box nucleolar RNA helicase, which presumably participates in aspects of RNA synthesis and processing (27). This finding is interesting because recent evidence suggests that, like topoisomerase II, Gu may be a target of ADR (28). However, the significance of this finding relative to HGF/SF-mediated cell protection remains to be determined.

A number of gene products implicated in signal transduction pathways were found to be up-regulated (e.g., RhoB and RAB5A) or down-regulated [e.g., STK2 (a serine/threonine kinase), PTPN2 (also known as T-cell protein tyrosine phosphatase, TCPTP) and 51C (also known as INPPL1 or SHIP-2)]. Expression of the *51C* gene, which encodes an the inositol polyphosphate-5-phosphatase (29), was decreased in both HGF/SF-treated cells (relative to control) and [HGF/SF + ADR]-treated cells (relative to ADR alone). This finding is of particular interest because of previous studies demonstrating a requirement for PI3K  $\rightarrow$  c-Akt signaling in the HGF/SF-mediated protection of breast cancer (MDA-MB-453) and glioma cell lines against apoptosis (6, 7, 30).

It had been reported previously that the tumor suppressor PTEN/MMAC1, an inositol polyphosphate-3-phosphatase, inhibited the PI3K/Akt pathway through its lipid phosphatase activity (15). Recently, *51C* was similarly found to act as an inhibitor of the PI3K/Akt pathway, presumably also by reducing the levels of phosphatidylinositol-3,4,5-phosphate [PI(3,4,5)P<sub>3</sub>], which is generated through the lipid kinase activity of PI3K (31). Thus, the reduced expression of *51C* in HGF/SF-treated cells should have the effect of maintaining the levels of PI(3,4,5)P<sub>3</sub>, which is essential for the activation and proper localization of c-Akt.

Interestingly, it has been demonstrated that one of the splice variants of the protein tyrosine phosphatase PTPN2/TCPTP, TC45, can inhibit epidermal growth factor receptor-mediated activation of PI3K/c-Akt signaling (32). Although the role of PTPN2 in c-Met receptor signaling and the important *in vivo* substrates for PTPN2 are unclear, the finding that HGF/SF down-regulates *PTPN2* gene expression again raises the possibility that PTPN2 is a target for the HGF/SF-mediated protection against DNA-damaging agents.

A cytoskeleton-associated cdc42-interacting protein, CIP4, was found to be up-regulated in ADR-treated cells, whereas HGF/SF blocked the up-regulation of CIP4. The function of CIP4 has not been

viability than those treated with ADR alone ( $P < 0.001$ , two-tailed  $t$  test). The viability of cells transfected with wtCIP4 and treated with [HGF/SF + ADR] was significantly reduced, compared with similarly treated untransfected or empty vector-transfected cells ( $P < 0.001$ ).

established definitively, but CIP4 may function, in part, to carry the Wiskott-Aldrich syndrome protein (WASP), a multidomain protein involved in cytoskeletal organization, from actin filaments to microtubules (10). We showed that forced expression of wild-type human CIP4 reduced the degree of HGF/SF-mediated protection of MDA-MB-453 cells to 50% or less of that observed in untransfected or empty vector-transfected control cells. On the other hand, expression of internally deleted or truncated CIP4 proteins did not inhibit cell protection. These findings suggest a role for CIP4 in cell survival/apoptosis pathways, a finding that is not obvious based on its known activities and protein interactions.

Although we have focused on some of the more novel findings of this study, not all of the cDNA microarray and RT-PCR results were unexpected. For example, the finding that ADR up-regulates c-Myc mRNA expression and that the up-regulation was blocked by HGF/SF was not unexpected. We reported similar results based on Western blotting of MDA-MB-453 cells (5). The transcription factor c-Myc has been implicated in a variety of cellular processes, including proliferation, differentiation, transformation, and apoptosis. Overexpression of c-Myc renders cells more susceptible to apoptosis through both p53-dependent and p53-independent mechanisms (33, 34). Thus, theoretically, down-regulation of c-Myc by HGF/SF in the setting of DNA damage might be expected to confer protection against apoptosis.

We had also reported that ADR down regulates the protein levels of the antiapoptotic protein Bcl-X<sub>L</sub>, whereas HGF/SF blocks the ADR-induced downregulation of Bcl-X<sub>L</sub> protein in MDA-MB-453 cells (5). Bcl-X<sub>L</sub> was not present among the cDNAs spotted onto the microarrays slides used in this study. However, we examined the Bcl-X<sub>L</sub> mRNA expression by semiquantitative RT-PCR analysis and found no ADR or HGF/SF alterations in Bcl-X<sub>L</sub> mRNA levels in multiple repeat assays. (6) Thus, the alterations in Bcl-X<sub>L</sub> protein levels probably occur through translational or posttranslational mechanisms. This finding suggests that some of the protection conferred by HGF/SF may be attributable to alterations in protein processing and metabolism. We had also noted that cell protection required a relatively long preincubation with HGF/SF of  $\geq 24$  h for some protection and  $\geq 48$  h for maximal protection (5). This consideration suggests that the ability of HGF/SF to block the reduction of Bcl-X<sub>L</sub> protein levels induced by ADR might be attributable to alterations in the expression of genes involved in the processing or metabolism of Bcl-X<sub>L</sub>.

Our findings suggest the viability of the cDNA microarray approach, coupled with additional studies to confirm gene expression alterations and functional studies to evaluate the significance of the findings, as a means of identifying novel and interesting genes that may be involved in HGF/SF cell protection pathways. It is likely that some of the genes for which expression was altered by HGF/SF in the setting of DNA damage are not involved in cell survival or apoptosis pathways. Alterations in these gene products may reflect other activities of HGF/SF than promotion of cell survival or may be a passive consequence of cell survival rather than a cause of survival. On the other hand, it is also likely that genes not implicated previously in cell survival or apoptosis mechanisms will be found to play roles in these processes (e.g., CIP4).

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Here is the information required for the submission of the manuscript to your journal:

1. The address of the corresponding author: Dr. P. M. R. Achary, Ullman-1219, Department of Radiation Oncology, AECOM, 1300, Morris Park Avenue, Bronx, NY-10461. Telephone: 718 430 2699; Fax: 718 430 2454; email: achary@aecom.yu.edu

2. Telephone numbers of all authors:

P. M. R. Achary, H. Zhao and Z. Fan: 718 430 2699; S. Gogineni and V. R. Pulijaal: 718 405 8100; L. Herbst: 718 430 8553; P. S. Mahadevia and J.G. Jones: 718 920 4976; H. P. Klinger: 718 430 2451; B. Vikram: 718 920 5280

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5. The main finding in this paper is the identification of a novel metastasis associated gene sequence (MAGS) in ductal breast carcinoma by representational difference analysis of DNA samples derived from microdissected normal and metastatic lymph node cells. PCR screening of MAGS-IX in 3 patient tumor tissue samples revealed that it is missing in 2 out of 3 positive lymph node cell DNA samples. RH mapping and FISH localized this MAGS on 10q21 of human chromosomes.

6. The following are the 5 keywords describing the manuscript:

i. Mammary carcinoma; ii. Metastasis; iii. Molecular markers; iv. Archival tumor samples; v. Representational difference analysis.

Thanks in advance for consideration of this manuscript.

Sincerely yours,



P. Mohan R. Achary Ph. D.  
Assistant Professor and Cancer Biologist



**Title:**

A candidate metastasis associated DNA marker for ductal mammary carcinoma<sup>1</sup>

**Authors and affiliations:**

P. Mohan R. Achary<sup>2</sup>, H. Zhao, Z. Fan, S. Gogineni, V. R. Pulijaal, L. Herbst, P. S. Mahadevia, Joan G. Jones, H. P. Klinger and B. Vikram

Metastasis Laboratory, Department of Radiation Oncology (PMRA, HZ, ZF, BV), Department of Obstetrics and Gynecology (SG, VRP) Department of Pathology (LH, JGJ, PSM) and Department of Molecular Genetics (HPK.), Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY 10461.

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<sup>2</sup>To whom requests for reprints should be addressed, Metastasis Laboratory, Ullman-1219, Department of Radiation Oncology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: (718) 430-2699; Fax: (718) 430-2454; email: achary@aecom.yu.edu

<sup>3</sup>The abbreviations used are: RDA, representational difference analysis; SCM, single cell microdissection; LCM, laser capture microdissection; RH, radiation hybrid; FISH, fluorescence in situ hybridization; MAGS, metastasis associated gene sequence.

## ABSTRACT

**Background:** Molecular genetic markers to identify the 13% lymph node negative mammary carcinomas that are prone to develop metastases would be clearly of considerable value in indicating those cases in need of early aggressive therapy. **Methods:** Representational difference analysis (RDA) was used in an attempt to identify genetic alterations related to breast cancer metastasis by comparing DNA from microdissected normal and metastatic cells of a ductal breast carcinoma patient. **Results:** One hundred cloned RDA products were examined and 10 unique metastasis associated gene sequences (MAGS), i.e. products apparently lost in metastatic cell DNA, were found. Of these MAGS, one sequence, MAGS-IX was found to be lost in the transition from primary to metastasis in 2 out of 5 ductal breast carcinoma cases. This sequence was localized on chromosome 10q21 by radiation hybrid mapping and fluorescence *in situ* hybridization. The PTEN gene which is also located on chromosome 10q was detected by PCR in all the five cases. On the other hand a breast carcinoma cell line, HCC-1937, which has homozygous loss of a region encompassing PTEN gene showed the presence of MAGS-IX. PCR screening of three additional breast carcinoma cell lines with known losses in specific chromosomal regions also showed the presence of MAGS-IX. **Conclusion:** These data suggest that MAGS-IX is a candidate molecular marker for ductal mammary metastasis.

## Introduction

The construction of a panel of molecular genetic markers for identifying the 13% lymph node negative mammary carcinomas that are prone to develop metastases [1] would be clearly of considerable value in indicating those cases in need of early aggressive therapy. There would also be considerable benefit to the 87% of women with mammary carcinomas who are not likely to metastasize by sparing them the physical, mental and financial costs of the treatment. In addition, further knowledge of the genetic mechanisms that play an important role in metastasis could ultimately lead to the development of improved therapeutic procedures.

While the evidence for the role of gene alterations in promoting metastasis in general, and in mammary carcinomas in particular, is still accumulating, it is very likely that like tumorigenicity suppressor genes and oncogenes, many more genes remain to be discovered that are involved in metastasis since the process is complex involving a large number of pathways [2]. Most of the known genetic mechanisms involved in the progression of a tumor to the metastatic state involve the loss of function of genes that prevent cells from becoming invasive. These are similar to tumorigenicity suppressor genes, whose loss of wild-type growth regulatory function leads to unregulated or malignant growth. Similarly the wild-type metastasis suppressor genes regulate the cell's mobility and its response to environmental messages, presumably keeping most cells localized and differentiated. Mutation or complete loss (deletion) of the wild-type counterpart of any such gene in a malignant cell may make that cell more motile, invasive



or metastatic. In particular, mutations of genes regulating cell adhesion molecules have been reported to be metastasis-fostering alterations [3, 4, 5, 6]. In human breast cancers, several other genes have been found to be associated with metastasis namely, nm23 [7, 8], KAI1 [9, 10], mta1 [11], KiSS1 [12], PTEN/MMAC1 [13, 14] and BRMS1 [15].

Representational difference analysis (RDA), a DNA subtractive hybridization method [16], was used in this study to test the working hypothesis that in order to achieve the metastatic state primary mammary carcinoma cells must acquire genetic changes in addition to those that led to transformation. With the RDA method, DNA from normal cells was compared with that of the metastatic cells of the same patient. The recovered differential sequences were then mapped on human chromosomes and used to screen DNA samples from normal, primary and metastatic cells of five additional ductal mammary carcinoma patients to determine if these were consistently associated with metastasis.

## **Materials and methods**

**Tissue samples and cell lines.** Normal breast tissue and lymph nodes with metastatic cells of a 52 years old patient (C-1050) with ductal mammary carcinoma were provided by the Co-operative Human Tissue Network (CHTN). Additional matched normal, primary and metastatic tissue samples were collected from four patients (C-18805; C-98-05H; C-19898; C-20635) from CHTN and one (DS-9605) from the Surgical Pathology Department of Albert Einstein College of Medicine and Montefiore Medical Center. The DNA samples from mammary carcinoma cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937) and matched normal cell lines were received from Dr. R. Parson of Columbia University, NY.

**Isolation of cells from biopsy samples by single cell microdissection (SCM).** Single cell microdissection was performed on hematoxylin and eosin stained tissue sections of positive lymph nodes from the ductal breast cancer patient samples (Fig. 1). In our method of SCM, a Zeiss axiovert phase contrast photomicroscope fitted with a TV monitor and a Narashige mechanical microdissector was used. A glass micro pipette was used to draw up individual cells after dissection. Tumor cells were identified in the microscope and using the tip of a glass micropipette attached to a syringe, they were dissected out without disturbing the surrounding tissue and then drawn into the pipette. After 5-10 cells were collected in the tip it was broken off and dropped into a sterile eppendorf tube. Approximately 10,000 metastatic tumor cells were collected in this manner for RDA experiments. As shown in figure 1, pure population of tumor cells were isolated from positive lymph node tissue samples. This method was used to isolate tumor cells for the RDA experiment because laser capture microdissection (LCM) equipment (see below) was initially not available.

**Isolation of cells from biopsy samples by laser capture microdissection (LCM).** LCM [17] was used for isolating tumor cells from primary and metastatic tissue samples of

captured on Nikon E800 using Quips Pathvysion system (Applied Imaging, Santa Clara, CA).

## **Results**

### **Isolation and characterization of candidate metastasis associated gene sequences (MAGSs).**

RDA was performed with the DNA recovered by SCM from normal cells and metastatic cells in the lymph nodes of a patient with ductal mammary carcinoma (Fig.1). Since the objective was to isolate sequences that are lost in the progression from normal to metastasis, we focused on the products of RDA in which the metastatic cell DNA was used as the driver and the normal cell DNA was the tester. As shown in figure 2, RDA differential products were isolated and DNA was extracted from each of the 5 bands obtained from the loss side of the third round of hybridization and DNA from each band was cloned separately. One hundred clones from each DNA band were saved and a subset of 100 clones (20 clones from each differential product) was selected randomly for further characterization. These 100 clones were probed (labeled with  $\alpha^{32}\text{P}$ -dCTP) on dot blots containing normal and metastatic cell DNA. It was found that 79 clones hybridized only with the normal cell DNA, and were not present in the metastatic cells (Fig. 3a). The remaining hybridized with both normal and the metastatic cell DNA. This may have been due to incomplete subtraction.

Of the 79 clones that hybridized only with the DNA of the normal cells, 50 were selected and tested further by Southern blotting to verify the RDA results. None of these hybridized with the DNA of the metastatic cells and all were detected in normal cells (Fig. 3b). These 50 clones were sequenced and those with identical sequences were grouped. Thus 10 unique candidate metastasis associated gene sequences (MAGS) were identified. The sequence homologies found by searching the nucleotide databases of these MAGS are presented in table 2. The search revealed 94-99% homology to known human gene sequences for nine of these MAGSs and one was found to be novel, having only limited homology with the sequences in the gene banks.

### **Results of Radiation Hybrid (RH) Mapping**

To determine if these 10 MAGS could be localized on human chromosomes, we used GeneBridge 4 Radiation Hybrid panel (Research Genetics, Inc.). For this physical mapping, and also to be able to use these sequences to screen additional patient samples, primer pairs were designed for each sequence (Table 2) and tested by PCR on a positive control (human genomic DNA) and two negative controls (Chinese hamster genomic DNA and no template). Of the 10 MAGS tested, only 5 (IV, VII, VIII, IX and X) were successfully mapped to specific human chromosomes (Table 2). Of those 5 MAGS, IV did not show any match with gene bank sequences and therefore appeared to be novel gene sequence. For MAGS VII, VIII, IX and X, the chromosome localizations obtained by physical mapping and by homology search agreed. The MAGS IX sequence showed 94% identity to a sequence on human chromosome 10. The last one, MAGS VIII was mapped to chromosome 6 with about 83% homology (Table 2).

### **Characterization of MAGS-IX by FISH and PCR**

We selected MAGS-IX for further characterization. Based on the sequence of the BAC clone (NCBI: RP11-407) encompassing MAGS-IX, a 2Kb DNA fragment containing MAGS-IX was generated by PCR. To localize MAGS-IX cytologically, we labeled the 2Kb sized MAGS-IX with Spectrum Green (Vysis) and hybridized on a normal human metaphase chromosomes. As shown in figure 4, MAGS-IX probe was hybridized on 10q21 region. Spectrum Green labeled centromere of chromosome 8 (CEP8; Vysis, Downers Grove, IL, USA) was used as a control. To determine if MAGS-IX is indeed associated with metastasis, DNA was used from cells recovered with the LCM method from normal, primary and metastatic tissue samples from 5 additional patients. These DNA samples were screened by PCR using primers designed for MAGS-IX (Fig. 5). MAG-IX was present in normal cell DNA samples of all the five patients but the intensity of hybridization was less in the primary tumor cell DNA samples from 2 patients and totally undetectable in the metastatic cell DNA of these two patient samples, suggesting a loss of this gene sequence during progression to metastasis. Because MAGS-IX was mapped to chromosome 10q21, we wanted to determine if it was located in the region encompassing a well-known MAG, PTEN [13]. We PCR screened DNA samples from normal, primary and metastatic lymph nodes of the above five patients with PTEN primers (Table 1) and found that PTEN is present in all of the samples (data not shown). To further determine if MAGS-IX is not a part of PTEN gene, we tested four pairs of cell lines derived from normal blood and tumor tissue samples of four patients with ductal breast carcinoma. While three tumor cell lines (HCC-1806, HCC-1143 and HCC-1428) are known to have loss of different chromosomal regions other than on 10q region [21, 22], the fourth tumor cell line (HCC-1937) is known to have homozygous loss for a region encompassing the PTEN gene [23]. PCR screening of the PTEN gene in these 4 tumor cell lines showed that it is present in the first three tumor cell lines and homozygously lost in the 4<sup>th</sup> cell line. PCR screening of MAGS-IX sequence in these cell lines however found to be present in all the tumor cell lines including HCC-1937 (Fig. 6). Consequently MAGS-IX appears to be lost independently of PTEN.

### **DISCUSSION**

In the present investigation we used representational difference analysis (RDA) in an attempt to identify genetic alterations related to breast cancer metastasis by comparing normal and metastatic cell DNA of a ductal breast carcinoma patient. Then differential products were used to screen DNA of five more sets of normal, primary and matched metastatic tumor samples (Fig.5). This analysis revealed one sequence, MAGS IX that was lost in the transition from normal to primary to metastasis in 2 of 5 cases (3 of 6 cases if the case used in the RDA assay is included). The fact that it was present in primary tumor cells, but missing in lymph node metastatic cell foci strongly suggests that this is a candidate marker for a novel metastasis suppressor gene.

The RH mapping revealed that MAGS-IX is localized to a 21cR interval between markers, D10S539 and D10S549, corresponding to human chromosome 10 band q21.1 (<http://ncbi.nlm.nih.gov>). This was also confirmed cytologically by using MAGS-IX as a FISH probe on human chromosomes (Fig. 4). To determine if this sequence is one of the

additional patients for screening candidate metastasis associated gene sequences by PCR. After many tumor cells have been captured on the LCM cap, it is then placed on a 500µl PCR tube containing ATL lysis buffer (Qiagen Co.) and the DNA is extracted.

**DNA extraction.** About 1µg of DNA was isolated from approximately 10,000 cells microdissected from metastatic tumor and normal (glandular and stromal) breast tissue samples, using a modified method of DNA extraction from archival tissues. Briefly, the microdissected cell pellet was incubated overnight in lysis buffer (Qiagen Co.) with Proteinase-K at 55°C. Glycogen (carrier) was added to the cell lysate and DNA was extracted using phenol-chloroform-isoamyl alcohol mixture followed by EtOH precipitation.

**RDA and characterization of differential products.** The RDA procedure was basically as described by Lisitsyn et al [16, 18] using Bgl II representation. The metastatic cancer cell DNA was used as the 'driver' and the DNA from the normal cells was the 'tester'. Three rounds of hybridization was performed to subtract the common sequences. The primers used for representation and hybridization steps of RDA are listed in Table 1. DNA from the differential bands (lost in metastasis) was cloned using the TA cloning system (pCR2.1; Invitrogen Co.). Clones were selected at random and used as  $\alpha^{32}\text{P}$ -dCTP (Amersham) probes on dot/Southern blots containing amplicon DNA from the original normal and metastatic cells to verify whether these sequences are actually present in normal and missing in metastatic cell DNA samples. Positive clones (showing signals in normal and missing in metastatic cell DNA) were sequenced and homology search performed using NCBI Blast program.

**Radiation Hybrid (RH) Mapping.** RH mapping was used to localize the MAGS on human chromosomes using high resolution Gene Bridge 4 radiation hybrid panels (Research Genetics, Inc.). Based on the sequences of differential products isolated from RDA experiments, primers were designed for each group of candidate gene sequences (Table 2). Based on the PCR results, chromosome localization of these sequences were determined using Whitehead Institute/ MIT databases (<http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper>).

**Fluorescence In Situ Hybridization (FISH) of MAGS-IX.** Chromosome preparations were made from PHA-stimulated peripheral blood lymphocyte cultures from normal individuals. The slides were fixed in methanol-acetic acid and air-dried. The probes used for FISH were Spectrum Green labeled centromere of 8 (CEP8; Vysis, Downers Grove, IL, USA) and Spectrum Green labeled 2Kb sized MAGS-IX sequence. Primers were designed from the BAC clone (NCBI: RP11-407) containing the MAGS-IX sequence to obtain 2Kb sized PCR product encompassing MAGS-IX region (Table. 1). The 2KB DNA fragment was run on a 1% gel, isolated from gel and eluted in water, labeled with Spectrum Green using nick translation method, following the manufacturer's instructions (Vysis). 3 days old slides were denatured in 70% formamide/2X SSC solution at 74°C. The dehydrated and air dried slides were used for hybridization according to published methods with some modifications [19, 20]. The slides were counter stained with DAPI (4, 6-diamidino-2-phenylindole dihydrochloride; Molecular Probes) and images were

commonly deleted regions in breast carcinomas, we screened normal and tumor derived cell lines from 3 patients which had loss in regions on different chromosomes and one had homozygous loss of a region encompassing PTEN gene [21, 22, 23]. While MAGS IX was present in the normal and tumor cell lines of all four patients, PTEN was present in three cell lines and absent in the 4<sup>th</sup> tumor cell line (Fig. 6) suggesting that MAGS-IX is neither a part of PTEN gene nor localized in the same deletion fragment that encompasses PTEN gene. Recent RT-PCR studies showed that MAGS-IX was an expressed sequence (data not shown). These results may not suggest MAGS-IX be part of a tumor suppressor gene but possibly a marker for loss of a functional gene located close by. Additional archival cases are being screened to determine if MAGS IX is a reliable marker for the metastatic potential of ductal breast cancers.

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## Tables

**Table 1.** Primers used in RDA and different PCR screening experiments.

Primer / Product size	Primer sequence (Forward)	Primer sequence (Reverse)
RDA representation	R24: 5'agcactctccagccttcaccgca3'	R12: 5'gatctgcggtga3'
RDA 1 <sup>st</sup> round hyb.	J24: 5'accgacgtcgactatccatgaaca3'	J12: 5'gatctgttcacg3'
RDA 2 <sup>nd</sup> round hyb.	N24: 5'aggcaactgtgctatccgagggaa3'	N12: 5'gatcttcctcg3'
RDA 3 <sup>rd</sup> round hyb.	Same as 1 <sup>st</sup> round (J24)	Same as 1 <sup>st</sup> round (J12)
PTEN (556)	5'ctcagattgccttataatagtc3'	5'tcatgttactgctacgtaaac3'
MAGS-IX (180bp)	5'aggtaggtagagtaacaggtttgtt3'	5'gatctgttctccctttcttagctt3'
FISH fragment (2Kb)	5'actatgtatgtgagcatctgtttgc3'	5'tgcactcttcacttgagtaaacttg3'
β-actin (295bp)	5'tcaccacactgtgccatctacga3'	5'cgacgtacacagcttctcctta3'

**Table 2.** The primers, the length, homology search and RH mapping results of the metastasis associated gene sequences (MAGSs).

MAGSs	Primers	Length (bp)	Homology	RH mapping
I	F. ATGCAGGAAGCGCTTGCTTGT R. GGACATCCTCACCATTGATCTC	205	Chromosome 5 (97%) AC005915.1	Not successful
II	F. CGAAGTTCCTGAATCAGTGGGATAT R. GATCTGAATGGTGAGGACGTCAGA	144	Chromosome 21 (99%) AC010463.6	Not successful
III	F. GTGAAGACGACAGAAAGGGCGTG R. GCAGTACCTCTGCAACACTGACG	277	Chromosome 1 (97%) AL359265.8	Not successful
IV	F. ATCTGTGTGTGCAGCGCTGTGGA R. GGAATCTCCGAGGACACTTGAGGT	185	No match	Chromosome 5; Places 19.72 cR from WI-6737
V	F. GGATGTCTTTCCATTGTTTAGGGC R. GGTCTTTCAGATGAATTCACAGATCT	220	Chromosome 16 (98%) AC004234.1	Not successful
VI	F. AAGAGAGGAAATATAGCAGTGGCAC R. GCTTACCGACGCGACTATCCATG	242	Chromosome 7 (97%) AC09333.1	Not successful
VII	F. CAGATAGGTGAGTGTATGTGAAGCA R. ATGGCTGTGCCCTTCTTCCCTA	190	Chromosome 6 (98%) AL121935.17	Chromosome 6; Places 1.51 cR from WI-3110
VIII	F. GCAATGTGAAAAGGCATATTTAGAAT R. GTCCACTGGCTGGTAATGGTGTA	170	Chromosome 6 (82%) AC013429.12	Chromosome 6; Places 3.67 cR from AFMA191WD1
IX	F. AGGTAGGTAGAGTAACAGGTTTGT R. GATCTGTTCTCCCTGGTCTTAGCTT	180	Chromosome 10 (94%) AC022541.10	Chromosome 10; Places 5.66 cR from D10S546
X	F. GATCTTCTCTCTCACAGCTCTGC R. ACCGACGTCGACTATCCATGAACA	192	Chromosome 15 (98%) AC104260.5	Chromosome 15; Places 1.71 cR from D15S157



## Legends

Fig.1. Single cell microdissection of tumor cells from fresh frozen breast tumor tissues. A tumor cell ( $\Rightarrow$ ) before (a) and after (b) microdissection. The arrows ( $\rightarrow$ ) showing cells for reference and ( $\Leftarrow$ ) showing intact collagen material (a) and those floating (b) on the section. Tumor cells in a positive lymph node tissue before (c) and after (d) microdissection.

Fig. 2. Isolation of differential products from single cell microdissected archival breast tissues. RDA hybridization was of the normal versus the metastatic tumor cell DNA and was performed in two ways. In the first RDA (Lane 2), the metastatic cell DNA was used as tester (which should yield differential sequences gained during malignant transformation or in the process of becoming metastatic) and in the second RDA (Lane 3), normal DNA was used as tester (which should yield sequences that were lost from the metastatic cells). In the third round of hybridization (c), the 'gain' lane contained 4 prominent DNA bands ranging from 200-300bp in size (c2) whereas in the 'loss' lane, there are 5 bands ranging from 200-370bp (c3) in size (arrows). Lane 1. Low molecular weight DNA marker (2Kb).

Fig. 3. Southern blot using MAGS-IX as an  $\alpha^{32}\text{P}$ -CTP probe on normal (N) and metastatic (M) amplicon DNA. Dot blot (a) and Southern blot (b) showing positive signals only in the normal cell DNA (N) but not in the metastatic cell DNA (M).

Fig. 4. Localization of 2Kb DNA fragment containing MAGS-IX on human metaphase chromosomes. Centromere of chromosome 8 was used as a positive control probe. MAGS-IX was found to localize around 10q21 chromosome region (close to centromere). Both the MAGS-IX and CEP-8 are labeled with spectrum green (Vysis) and the chromosomes were counter stained by DAPI.

Fig. 5. PCR screening of MAGS IX on normal (N), primary (P) and metastatic (M) cell DNA samples of 3 patients (1st Patient: lanes 1, 2 and 3; 2nd Patient: lanes 4, 5 and 6; 3rd Patient: lanes 7, 8 and 9). Results show the target DNA band (148bp size) is missing in the metastatic cell DNA of patients 1 (lane 3) and 3 (lane 9).  $\beta$ -actin was as an internal control.

Fig. 6. PCR screening of MAGS IX and PTEN on DNA samples from 4 tumor cell lines and matched normal DNA. Results (first row) show that MAGS-IX is present in all the tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937) especially the 4<sup>th</sup> tumor cell line which was known to have loss of a region encompassing the PTEN gene. Screening of PTEN in the above four cell lines (second row) show its presence in three tumor cell lines but as expected is missing in the 4<sup>th</sup> cell line (HCC-1937).  $\beta$ -actin was as an internal control.

## Illustrations

(6 figures enclosed)

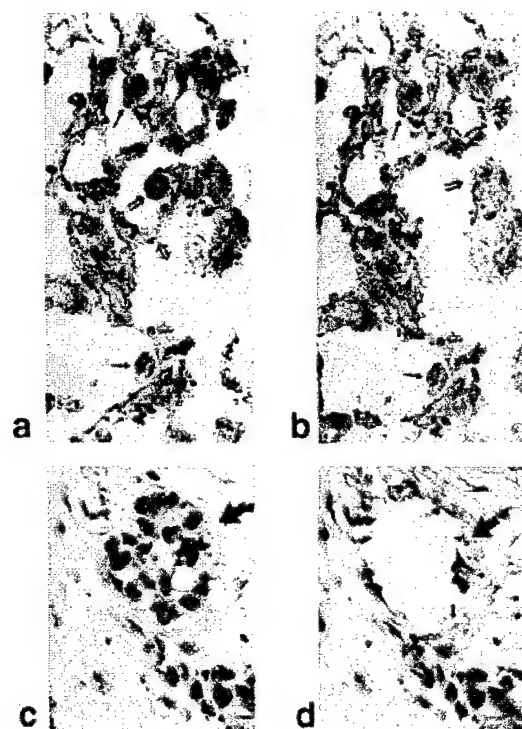


Fig. 1

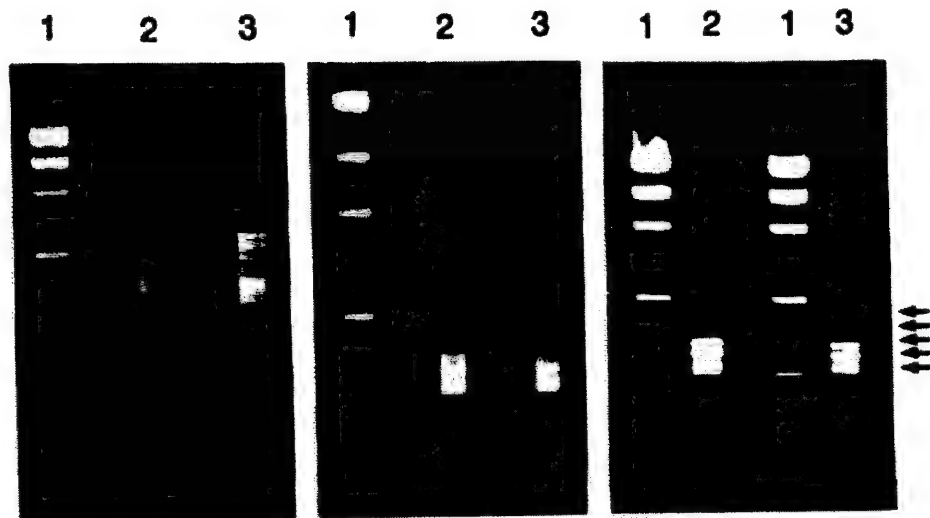
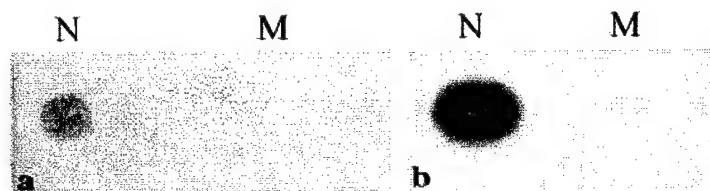


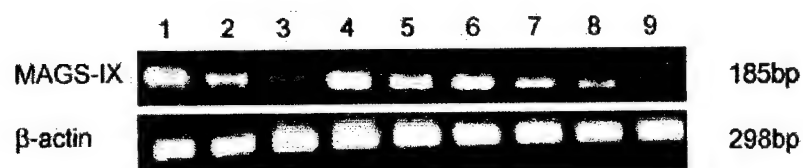
Fig. 2



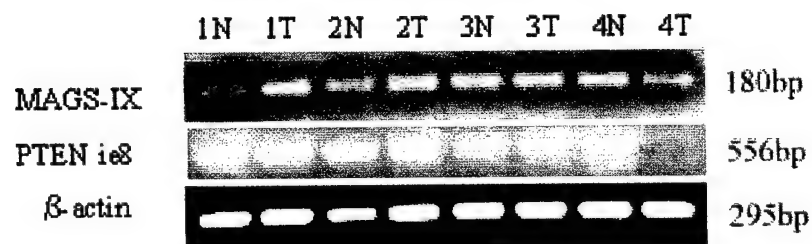
**Fig. 3**



**Fig. 4**



**Fig. 5**



**Fig. 6**

**Dr. Herbert E. Nieburgs,  
Editor-In-Chief,  
Cancer Detection and Prevention.  
University of Massachusetts Medical Center  
55 Lake Avenue North, Box 20  
Worcester, MA 01655 USA**

July 30th, 2002.

Dear Dr. Nieburgs:

I enclose herewith a manuscript entitled "Microsatellite dinucleotide (T-G) repeat: A candidate DNA marker for breast metastasis." for consideration of publication in your journal, "Cancer Detection and Prevention".

Here is the information required for the submission of the manuscript to your journal:

1. The address of the corresponding author: Dr. Mohan P. Achary, Ullman-1219, Department of Radiation Oncology, AECOM, 1300, Morris Park Avenue, Bronx, NY-10461. Telephone: 718 430 2699; Fax: 718 430 2454; email: achary@aecom.yu.edu

2. Telephone numbers of all authors:

B. Mukherjee, Mohan P. Achary, H. Zhao, B. Parashar and B. Vikram : 718 430 2699; P. S. Mahadevia: 718 920 4976; H. P. Klinger: 718 430 2451; B. Sood: 718 670 1231.

3. That this manuscript has been read by all authors and in part or full has not been communicated or published in any journal and all the authors are aware and agree the content of the paper.

4. The main finding in this paper is the identification of a metastasis associated DNA sequence (MADS-XI) in ductal breast carcinoma. DNA samples were isolated from normal and metastatic lymph node cells microdissected by single cell microdissection method. Slot Blot screening of MADS-XI in 5 patient tumor tissue samples revealed that it is missing in 4 out of 5 positive lymph node metastasis cell DNA samples suggesting its possible use as a diagnostic molecular marker for breast metastasis.

5. The following are the keywords describing the manuscript:

Key Words: Dinucleotide repeat, Metastasis, Metastasis Associated DNA Sequence (MADS), Representational Difference Analysis (RDA).

6. Suggested reviewers:

a) Dr. M. Jhanwar-Uniyal, Ph.D.  
Scientist 4P  
American Health Foundation,  
1, Dana Road, Valhalla, NY 10595.  
Ph. No. (914)-789-7135.  
E-mail: Mjhanwar@aol.com

b) Dr. Richard A. Britten, Ph.D.  
Associate Professor,  
Dept. of Radiation Oncology,  
Eastern Virginia Medical School,  
600 Gresham Drive,  
Norfolk, VA.23507-1999.  
Tel: (757)-446-5038.  
E-mail: BritteRA@EVMSMAIL.EVMS.EDU

c) Dr. Linda A. Cannizzaro, Ph.D.  
Professor,  
Dept. of Pathology,  
Montefiore Medical Center,  
111E 210th Street,  
Bronx, NY 10467.  
Tel: (718)-920-7929.  
E-mail: cannizza@acom.yu.edu

Thanks in advance for consideration of this manuscript.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Mohan P. Achary', with a stylized flourish at the end.

Mohan P. Achary Ph. D.  
Assistant Professor and Cancer Biologist  
Albert Einstein College of Medicine

**Microsatellite dinucleotide (T-G) repeat: A candidate DNA marker for breast metastasis.**

Bhaskar Mukherjee, Ph.D,<sup>a</sup> Hui Zhao, Ph.D,<sup>a</sup> Bhupesh Parashar, M.D,<sup>a</sup> Brij M. Sood, M.D,<sup>a</sup> Panna S. Mahadevia, M.D,<sup>b</sup> Harold P. Klinger, M.D., Ph.D,<sup>c</sup> Bhadrasain Vikram, M.D,<sup>a</sup> Mohan. P. Achary, Ph.D<sup>a</sup>

**Running Title:**

Markers for breast metastasis.

**Author Affiliations:**

<sup>a</sup>Metastasis Laboratory, Department of Radiation Oncology, <sup>b</sup>Department of Pathology and <sup>c</sup>Department of Molecular Genetics, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY 10461.

**Correspondence and reprint request to:**

Mohan. P. Achary, Ph.D.  
Metastasis Laboratory, Department of Radiation Oncology  
Albert Einstein College of Medicine, Bronx, NY 10461  
Tel # (718)-430-2699.  
Fax: # (718)-430-2454.  
E-mail: achary@aecom.yu.edu.

**Abstract:**

A dinucleotide (T-G) repeat sequence was isolated by comparing DNA from metastatic lymph node and matched normal breast samples from a ductal mammary carcinoma patient using representational difference analysis (RDA) method. Our present study used this metastasis associated DNA sequence (MADS) as a diagnostic probe to screen five patient samples by slot blot method. A new approach to isolate single cells by microdissection, namely single cell microdissection (SCM) was developed to obtain homogeneous population of tumor cells (~1000) from matched primary tumors and corresponding positive lymph nodes of 5 patients. We isolated DNA from these homogeneous tumor cells and used for the RDA and DNA slot blot experiments. The screening of patient samples showed loss of this MADS in the transition from primary to metastasis in 4 out of 5 cases (80%) suggesting its possible role in breast metastasis.

**Key Words:** Dinucleotide repeat, Metastasis, Metastasis Associated DNA Sequence (MADS), Representational Difference Analysis (RDA).



## **Introduction:**

The dissemination of cancer cells from primary site is the main cause for relapse of disease in patients with solid tumors. The degree of spreading of tumor cells in the circulation involves multiple tumor-host interactions through complex genetic mechanisms. Alterations in the expression of both the oncogenes and tumor/metastasis suppressor genes are known to be involved in metastasis<sup>1-3</sup>. Mutation or complete loss (deletion) of the wild-type counterpart of any suppressor gene in a malignant cell may increase cell motility, invasion or metastasis. Metastasis is a complex process involving a cascade of biochemical and genetic events that regulate growth, vascularization, invasion, transport and survival in the circulation followed by adhesion, extravasation, and proliferation at the distant sites<sup>3</sup>. In human and mouse breast cancers only few genes associated with metastasis have been reported<sup>4-7</sup>.

We used the RDA method<sup>8-10</sup> in an attempt to identify candidate DNA marker sequences and/or genes involved in the progression of the disease from benign to invasive breast carcinoma stage to metastasis by comparing DNA samples from pure population of microdissected tumor cells from positive lymph nodes and matched normal cells from the same patient. Southern blots showed that most of the sequences (87%) were present in normal cell DNA and partially or completely missing in the metastasis cell DNA samples<sup>10</sup>. We found that one of these metastasis associated DNA sequences (MADS-XI) is enriched with novel dinucleotide (T-G) repeats. Since microsatellite repeats are known to regulate the expression of certain genes associated with metastasis<sup>11-13</sup>, we made an attempt to determine if this novel gene sequence, MADS-XI could be used to predict the primary tumors that are prone to develop metastasis. We therefore screened additional 5 patient samples by DNA slot blot method to determine if this MADS-XI is actually involved in metastasis to lymph nodes in breast carcinoma.

## **Materials and Methods:**

**Tissue Samples:** Archival tumor tissue material from 5 patients with ductal mammary carcinoma (coded case numbers: 1: C-14153; 2: C-1050; 3: DS97-919; 4: C-14852; 5: DS-711) were obtained from Co-operative Human Tissue Network (1, 2 and 4) and from the Surgical Pathology Department of Montefiore Medical Center (3 and 5). Each case consisted of primary and metastatic lymph node tumor tissue samples and matched normal tissue from the same patient.

**Isolation of cells from biopsy samples by single cell microdissection:** Single cells from tumor biopsy samples were isolated using an innovative microdissection approach namely single cell microdissection (SCM). Thus homogeneous populations of tumor cells (~1000) were isolated from matched primary tumors and corresponding positive lymph nodes of patient samples. SCM was performed on hematoxylin and eosin stained tissue sections from primary tumors and metastatic lymph nodes from ductal breast carcinoma patient samples. In our method of SCM, a Nikon inverted photomicroscope fitted with a

Narashige mechanical microdissector was used (Fig. 1). Tumor cells were identified in the microscope and using the tip of a glass micropipette attached to a syringe, they were dissected out one after the other without disturbing the surrounding tissue and then drawn into the pipette (Fig. 2a and b). After 5-10 cells were collected in the tip it was broken off and dropped into a sterile eppendorf tube. As shown in figure 2 (c and d), pure population of tumor cells was isolated from a positive lymph node tissue sample. Compared to laser capture microdissection methods, this is less expensive and provides high degree of homogeneity of cell population compared to other methods. SCM method also alleviates any possible damage to the DNA or RNA of the microdissected cells as UV or infra red sources of laser beams are not used in this method. Since the collection of large number of homogeneous cells by this method is very tedious and time consuming, we have modified RDA protocol and standardized a genomic DNA amplification method to isolate larger amounts of DNA from relatively few cells (~1000) for routine DNA slot blot<sup>14</sup> preparations.

**DNA extraction:** About 100ng of DNA was isolated from approximately 1000 cells microdissected from primary tumor and metastatic lymph node tissue samples, using modified methods of DNA isolation and genomic DNA amplification from limited cells. Briefly, the microdissected cell pellet was incubated with Proteinase-K (1 $\mu$ l of 20 $\mu$ g/ml stock) in a 20 $\mu$ l of Tris- buffer for one hour at 37°C. The sample was denatured at 95°C for 2 mins, cooled to room temperature and BglII digestion (1:200 dilution of the stock 10U/ $\mu$ l enzyme) was set up using 1 $\mu$ l of the diluted enzyme in a 20 $\mu$ l reaction mixture containing 100ng of DNA. The digestion was carried for two hours and the DNA fragments were ligated with the 24mer and 12mer oligos and BglII amplicons were prepared<sup>8,11</sup>. PCR amplification of these BglII representation amplicons was performed using 24 mer oligo primers (95°C 1min, 72°C 3min. for 30 cycles followed by 1 cycle at 72°C for 10 min.). DNA was extracted using phenol-chloroform-isoamyl alcohol mixture followed by EtOH precipitation. The DNA samples were quantified on an agarose gel using a high mass DNA maker (0.1 to 10Kb) and predetermined quantities of sheared salmon sperm DNA (0.1, 0.2, 0.5, 0.7 and 1 $\mu$ g).

**RDA and characterization of differential products:** The RDA procedure was basically as described by Lisitsyn et al.<sup>8,9</sup> using Bgl II representation. The metastasis cancer cell DNA was used as the 'driver' and the DNA from the normal cells was the 'tester'. Three rounds of hybridization were performed to subtract the common sequences<sup>8-10</sup>. DNA from the differential bands (lost in metastasis) was cloned using the TA cloning system (pCR2.1; Invitrogen Co.). Clones were selected at random and used as <sup>32</sup>P-dCTP (Amersham) probes on dot/Southern blots containing DNA from the original normal and metastasis cells to verify whether these sequences are actually present in normal and missing in metastatic cell DNA samples<sup>10</sup>. Positive clones (showing signals in normal and missing in metastatic cell DNA) were sequenced and homology search performed using NCBI Blast program.

**Slot-blot Method:** The slot blot method was performed following published methods<sup>14</sup>. Briefly, the PCR amplified products were purified by phenol chloroform method as mentioned elsewhere and suspended in 50 $\mu$ l of TE buffer. One microgram of the

quantified product was denatured with NaOH (1N) and incubated for 5min. at 37°C. The final volume was made up to 100ul with 10XSSC (final concentration of 6XSSC). The samples were applied on pretreated nitrocellulose membranes using S&S (Schleicher & Schuell) Minifold II slot-blotter apparatus following manufacturer's instructions. The membrane was UV cross-linked (Stratalinker 2400) and pre-hybridized for three hours followed by one-hour hybridization with the quick hybridization solution (Stratagene, CA). The <sup>32</sup>P dCTP labeled MADS-XI/GAPDH probe was made and purified as per manufacturer's protocol (Stratagene). The hybridization solution contained about 2x 10<sup>6</sup> cpm/ml of the probe. The filter was then washed and exposed to X-ray film for 1-3 hours and developed for autoradiography. Blotting experiments were repeated twice and the reproducible results were analyzed.

### Results:

The MADS-XI (224bp) has a 68bp unique sequence followed by a 156bp sequence with 17 groups of varying number of repeats, interrupted by 1-10 nucleotides (Fig. 3). Blast and Blat search results showed high homology (98%) of this sequence with regions on chromosomes 6q. However when only dinucleotide repeats sequence (without the unique sequence) was blasted for homology search it showed high homology with the dinucleotide repeats on chromosomes 6, 16 and 17. Physical localization using radiation hybrid mapping however was not successful due to several non-specific PCR products with human and chinese hamster ovary (CHO) template DNA samples.

Slot blot analysis of DNA from primary tumor cells and metastatic lymph nodes from 5 patients with ductal mammary carcinoma using the MADS-XI as a probe revealed its involvement in breast metastasis. As shown in the figure 4, the MADS-XI was present in the normal cells from all the 5 patients while missing in 4 out of 5 (80%) metastasis cell DNA samples. In the patient number 3, however, the primary cell DNA band showed lesser (half) intensity suggesting the loss of heterozygosity (LOH).

### Discussion:

Dinucleotide repeats are known to regulate the expression of several tumor/metastatic suppressor genes<sup>11,13</sup>. The loss of this MADS-XI in metastatic lymph node cell DNA samples from 4 out of 5 patients suggests its involvement in breast metastasis (Fig. 4). The reduction in the intensity of signal to about half from the normal to primary tumor cell DNA (Fig 4a; Case number 3) and complete loss in lymph node metastasis may suggest that this sequence can be used as a diagnostic tool for breast lymph node metastasis. These findings are in support of our hypothesis (Fig 5) that gradual loss of T-G repeats in tumor cells is associated with the progression of the disease which leads to the instability (MSI) and/or loss of several tumor/metastasis suppressor genes in metastasis.

Blat search results indicated that both the whole MADS-XI sequence and only T-G repeat sequence from MADS-XI is matching with a sequence from chromosomes 6,16 and 17 with high scores (98.7%, 92%, 93%). The unique sequence (68bp) from MADS-XI,

without T-G repeat sequence has matched only with chromosome 6q (98%). This may suggest that microsatellite instability (MSI) due to loss of repeat sequences may cause allelic instability (AI) in several different chromosomes simultaneously. Our results implicate involvement of at least three chromosome regions in breast lymph node metastasis. They might also influence some important genes located in these three chromosomes [IGF2R/M6PR; 6q26, 6q27]<sup>15,16</sup>, [p53; 17p13.1]<sup>17,18</sup>, [BRCA1; 17q12-q21]<sup>19</sup>. Others also have reported the allelic loss of chromosomes 6, 16 and 17 because of MSI and AI during disease progression<sup>20-23</sup>. Besides, MSI causes replication error mismatch repair induction followed by mismatch repair gene mutation. Thus sum of these events probably activate the protooncogenes and inactivate tumor/metastasis suppressor genes leading to metastasis<sup>24</sup>.

Currently we are screening circulating tumor cells with MADS-XI alone and in combination with other known makers to evaluate the state of the breast disease. The prediction for the genetic status of the circulating tumor cells will eventually convey the guidelines for early stage therapeutic modality to arrest or delay the progression of the disease<sup>25</sup>.

#### **Acknowledgements:**

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### Legends to figures 1 to 5

Fig. 1. Single cell microdissection apparatus. An inverted tissue culture Nikon photo microscope attached with a Narashige micromanipulator for single cell microdissection. A capillary glass micropipette is fitted to the micromanipulator needle holder to dissect the tumor cells and a syringe is connected with a plastic tubing to the other end of the micropipette to draw tumor cells into its tip.

Fig 2. Single cell microdissection of tumor cells. Dissection of one tumor cell from the primary tumor section (a: before and b: after) and a group of tumor cells from the positive lymph node (c: before and d: after) of a ductal mammary carcinoma.

Fig. 3. The complete MAGS-XI sequence enriched with TG repeats. Bold letters: Unique sequence; V: Vector sequence

FIG. 4. Slot blot analysis of DNA samples from primary tumor and metastatic lymph node samples and matched normal cells from 5 patients (1: C-14153; 2: C-1050 3: DS 97-919; 4: C-14852; 5: DS-711). The first (N), second (P) and third (M) rows represent the DNA samples from normal, primary tumor and metastatic lymph node breast samples respectively. (a) The blot is hybridized with  $^{32}\text{P}$  dCTP labeled MADS XI probe; (b) The DNA loading is quantitated in the experimental blot by probing with GAPDH.

Fig. 5: Schematic representation of loss of T-G repeats with the progression of the disease. (a) Normal cells with  $[(\text{T-G})_n]^+$  repeats; (b) Primary cells with  $[(\text{T-G})_n]^+$  and without  $[(\text{T-G})_n]^-$  repeats and (c) Metastatic cells without any repeats  $[(\text{T-G})_n]^-$ .

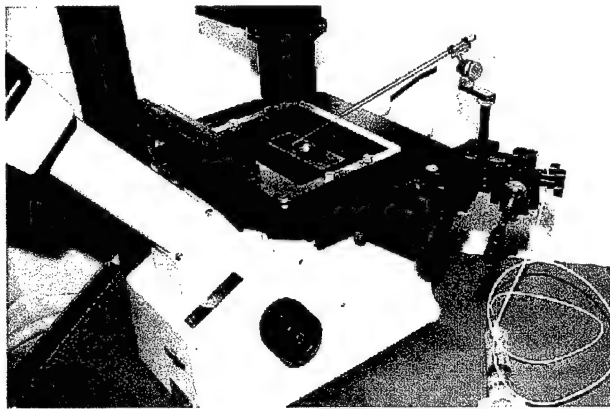


FIG: 1

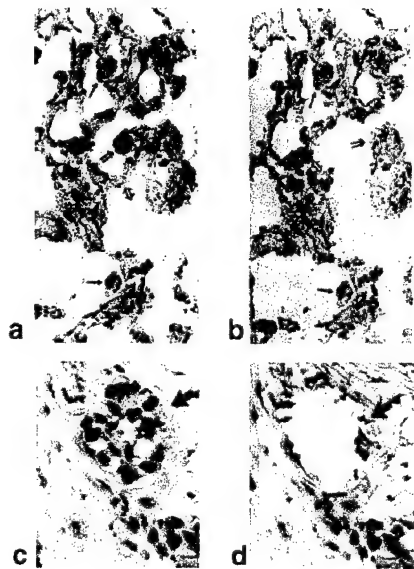
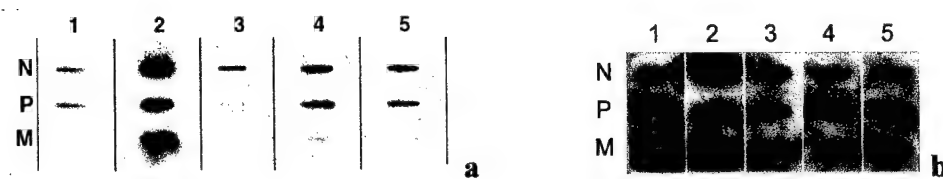


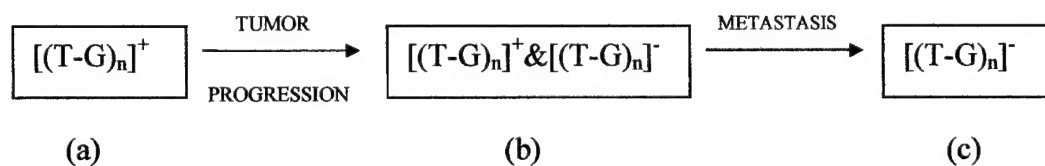
FIG: 2

**VGATCTTAATCCGGGGAGTGGCGTATGTAGTAGAAGAGTCTGGATTGAGTAG**  
**TGTATGGTAACGCCAG (T-G)<sub>5</sub> C (T-G)<sub>4</sub> GTA (T-G)<sub>2</sub> G (T-G)<sub>4</sub> T (T-G)<sub>3</sub> GCA (T-G)<sub>3</sub> G (T-**  
**G)<sub>2</sub> TATGG (T-G)<sub>3</sub> G (T-G)<sub>3</sub> A (T-G)<sub>4</sub> GTGCG (T-G)<sub>2</sub> AGATACGTGG (T-G)<sub>3</sub> GGG (T-G)<sub>6</sub> G (T-**  
**G)<sub>3</sub> G (T-G)<sub>5</sub> GTA (T-G)<sub>2</sub> G (T-G)<sub>3</sub> GV.**

**FIG: 3**



**FIG : 4**



**FIG : 5**

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**ABSTRACTS**

December 4, 2001

Albert Einstein College of Medicine  
of Yeshiva University

Belfer Institute for Advanced Biomedical Studies

## Characterization of Metastasis Associated Gene Sequences in Breast Carcinoma

H. Zhao, Z. Fan, L. Herbst<sup>1</sup>, H. P. Klinger<sup>2</sup>, B. Vikram and P. Mohan R. Achary  
*Metastasis Laboratory, Departments of Radiation Oncology, Pathology<sup>1</sup>, Molecular Genetics<sup>2</sup>,  
Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.*

**About** 13% mammary carcinoma patients with negative lymph nodes, even though treated with surgery or surgery and radiation therapy, are still prone to developing metastasis. The objective of this project is to construct a panel of molecular genetic markers for detecting those 13% patients so that they could be treated more aggressively. This is also beneficial to the remaining 87% patients who are not likely to metastasize. These women could then be treated more conservatively, sparing them the considerable physical, mental and financial costs of the treatment and with great ease of mind. The long-term goal of this study is to use these markers for better prognosis as well as the basis for the development of novel therapies in future.

**The** hypothesis to be tested is that in order to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic changes in addition to those that led to transformation. This may involve the loss of function of metastasis suppressor genes or the activation of metastasis promoting genes such as oncogenes. The existence of both types of genes have been reported in several tumor types, but many more are very likely to exist, and much remains to be learned about the possible roles of such genes in mammary carcinoma. A subtractive DNA hybridization technique, Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumor with that of the metastatic lymph node of the same patient to isolate those sequences that were lost in the course of tumor metastasis. The tumor and metastatic cells were recovered by laser capture microdissection.

**We** have isolated 11 candidate metastasis associated gene sequences (MAGS) that were found to be lost in metastatic cells. When screened on normal, primary and metastatic cell DNA samples from 5 breast carcinoma patients one of them (MAGS-XI) was found to be lost in the metastatic cells of 3 out of 5 patients and another sequence (MAGS-IX) in 2 out of 3 patients indicating their involvement in breast metastasis. Presently we are isolating partial or full-lengths of these MAGS using inverse PCR method in order to use these sequences as fluorescence *in situ* hybridization (FISH) probes to screen large number of patient samples.

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were identified using DGGE and characterized by sequencing (Clin Cancer Res. 2000; 6:3923). For p53, immunohistochemical staining (DO-7 monoclonal antibody, DAKO) was considered positive if observed in 25% or more of the tumor cells. Tumors were counted as HER2 overexpressing when a strong staining of the entire membrane (c-erbB-2 antibody, DAKO) was observed in more than 10% of the tumor cells ('3+' in the HercepTest guidelines). All patients were treated according to the Danish Breast Cancer Cooperative Groups guidelines for the DBCG 89 protocols. **Results:** The study included 428 patients, 205 node-negative and 223 node-positive. TP53 mutation was found in 25%, p53 expression in 48%, and HER2 overexpression in 18%. Except for null mutations, most tumors with TP53 mutations had p53 expression. However, p53 expression was also observed in 42% of TP53 wild-type tumors. TP53 mutation, HER2 overexpression, and to a lesser degree p53 expression, was associated with parameters related to tumor aggressiveness (positive lymph nodes, negative receptor status, high degree of anaplasia etc). Univariate analysis showed that disease-specific survival was correlated to tumor size, nodal status, degree of anaplasia, estrogen receptor status, TP53 mutation, p53 expression, and HER2 overexpression. When analyzed according to nodal status, TP53 mutation and HER2 overexpression, but not p53 expression, significantly correlated with poor survival probability in each of the subgroups. A Cox proportional hazard analysis including all 428 patients demonstrated that positive nodal status (1-3 positive nodes: relative risk (RR) 1.6, 95% CI: 1.0-2.7, and >3 positive nodes: RR 4.2, 2.7-6.3), TP53 mutation (RR 2.0, 1.4-3.0), and HER2 overexpression (RR 2.6, 1.8-3.8) were the only parameters which had independent poor influence on reduced disease-specific survival. TP53 and HER2 retained their independent poor influence on survival when analyzed according to nodal status. Same patterns were observed for overall survival. **Conclusion:** TP53 mutation and HER2 overexpression are strong markers for the prediction of disease-specific and overall survival in early breast cancer, irrespective of nodal status. p53 expression is only a weak marker, and its significance is lost when TP53 mutational analysis is included.

**#217 Correlation of established and novel molecular biology markers (E&NMBM) with long-term outcome / disease biology of stage I-III breast cancer (BrCA) patients (pts).** Multicentre collaboration of the British Columbia Breast Cancer Tissue Array Project (BCBCTAP). Joseph Ragaz, Torsten Nielsen, Marc Lippman, Mat Van de Rijn, Forrest Hsu, Stephen Chia, Angela Brodie, George Sledge, Jr., Adrian Harris, Shoukat Dedhar, Malcolm Hayes, Caroline Speers, John Spinelli, Douglas Ross, Charles Perou, David Huntsman, and Blake Gilks. BC Cancer Agency, Vancouver, BC, Canada, University of British Columbia, Vancouver, Canada, University of Michigan, Ann Arbor, MI, Stanford University, Stanford, CA, BC Cancer Agency, Vancouver, Canada, University of Maryland, Baltimore, MD, Indiana University, Indianapolis, IN, and Churchill Hospital, Oxford, UK.

Since the late 1970's, 2,154 pts participating in five British Columbia BrCA trials involving stage I-III breast cancer have been prospectively followed. Of those, 932 had their pathology material and paraffin blocks recovered, and tissue array blocks built. The following main cohorts at different risk considered to reflect different disease biology, and their 10 year overall survival (OS) rates, were identified. Also, subsets of node positive patients with extensive nodal/extracapsular spread (EN/ES+ve) with significantly worse outcome were identified (10 y OS of EN/ES+ve vs EN/ES-ve pts: 50 vs 70%,  $p < 0.001$ ). These results reflect disease of different risk and biology. In the newly started BCBCTAP, each risk category will be analyzed for expression of E&NMBM as determined by tissue array methodology. The novel markers will include those recently identified by cDNA microarray (Nature 2000, 406:747-52 MvR, DR,CP); EGF-receptor variants and ErbB1/2 (ML); integrin linked kinase and matrix metalloproteinases (SD); aromatase and the Cox2 pathway (AB); Carbonic Anhydrase & markers of hypoxia (SC, AH); p-glycoprotein / MDR, and cyclin D/Ki67/Bcl2 pathways (MH); VEGF (GS); and EMSY, a newly identified gene amplified in many breast cancers and associated with BRCA-1 (DH). Their multivariate interaction with the conventional pathology markers & the 20 year outcome of above patients, as well the proposal for more collaboration with screening of novel genetic markers on additional BrCA pts from the BCBCTAP, will be presented.

	10 year OS (%)
Node (-)	81.5%
Node (+)	58.5%
Stage III	40.1%
Inflammatory	13.8%

**#218 Expression of BP1, a homeobox gene, strongly correlates with ER expression in breast cancer.** Patricia E. Berg, Arnold Schwartz, Holly Stevenson, Gregory Davenport, Jan M. Orenstein, Peter Guterres, and Sidong Fu. George Washington University Medical Center, Washington, DC, Dynport Vaccine Company, LLC, Frederick, MD, and University of Maryland Medical School, Baltimore, MD.

We have cloned a potential new human oncogene termed BP1 which contains a homeobox and is a member of the Distal-less (DLX) family of homeobox genes. BP1 expression was examined in tissues from 29 newly diagnosed breast cancer patients. Overall, 66% expressed high levels of BP1 mRNA. BP1 expression was seen in 92% of the high grade, estrogen receptor (ER) negative, progesterone receptor (PR) negative cancers, but in only 40% of ER positive, PR positive breast

cancers. In contrast, BP1 was expressed at a very low level in only one of six normal breast tissues. Interestingly, all trans-retinoic acid (ATRA), a retinoid used therapeutically to treat breast cancer, was found to repress BP1 in MCF7 cells. Of potential relevance to breast cancer, BP1 maps near BRCA1. Supporting the oncogenic potential of BP1, we previously showed that BP1 mRNA is overexpressed in 63% of adult acute myeloid leukemia (AML) patients, although it is barely detectable in normal bone marrow. Ectopic expression of BP1 led to increased survival of K562 leukemia cells, while reducing BP1 expression caused apoptosis. Therefore, BP1 appears to be part of an anti-apoptotic pathway, suggesting a general mechanism by which BP1 could function as an oncogene. Our analysis of breast tumors suggests that BP1 may be a new marker in breast cancer for poor prognosis tumors and that it is a potential molecular target for therapy, an idea supported by BP1 repression by ATRA.

**#219 Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer.** Lin Wang, Robert Z. Luo, Jinsong Liu, Aysegul A. Sahin, Amanda McWatters, Robert C. Bast, Jr., and Yinhua Yu. The University of Texas M D Anderson Cancer Center, Houston, TX.

Ductal carcinoma in situ (DCIS) is an early, localized stage of multistep breast carcinogenesis that accounts for approximately 20-25% of mammographically detected breast cancers. A significant fraction of untreated DCIS will evolve into invasive cancer. ARHI is an imprinted tumor suppressor gene that is expressed in normal breast epithelial cells but that is down-regulated in a majority of breast cancers. In order to investigate the relationship of ARHI expression to the progression of breast cancer, we examined ARHI expression in 50 formalin-fixed and paraffin-embedded DCIS specimens from the Breast Cancer Tissue Bank at M. D. Anderson Cancer Center. Normal breast epithelium was found in 43 specimens and invasive breast carcinoma was found in 20 specimens. Both immunohistochemistry and in situ hybridization were used to evaluate ARHI expression. Prior to immunohistochemical assays, tissue sections were steam heated for 3 min to enhance antigen expression. After incubation with a murine monoclonal anti-ARHI antibody, ARHI was detected with biotin-streptavidin peroxidase display. Antibodies against Leukocyte Common Antigen were used as a negative control. For in situ hybridization, an ARHI mRNA antisense probe was employed to detect the ARHI gene. An ARHI mRNA sense probe was used as a negative control and the housekeeping gene GAPDH mRNA antisense probe was used as a positive control. ARHI mRNA and protein were identified in all normal breast epithelia. ARHI expression was concentrated in cytoplasm and rarely present in the nucleus. Compared to adjacent normal breast epithelia, ARHI protein expression was down-regulated in 35% (15/43) of DCIS and 65% (13/20) of invasive carcinomas. When DCIS and invasive cancer were present in the same sample, ARHI was further down-regulated in invasive carcinoma by 40% (6/20). In two cases (2/20, 10%) of invasive carcinoma, ARHI protein expression was totally lost. Consistent results were obtained with the in situ hybridization. Our study indicates that a decreased ARHI expression is associated with progression of breast cancer.

**#220 A candidate metastasis associated genetic marker for ductal mammary carcinoma.** Hui Zhao, Zuohe Fan, Lawrence Herbst, Dwayne Breining, Joan G. Jones, Panna S. Mahadevia, Harold P. Klinger, Bhadransai Vikram, and Mohan Rao P. Achary. Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY, and Albert Einstein College of Medicine and Montefiore Medical Center.

Metastasis is responsible for most deaths from breast cancer. The objective of this project is to identify a panel of molecular genetic markers for detecting those 13% of mammary carcinoma patients with negative lymph nodes that are prone to developing metastasis even after surgery, or surgery combined with radiation and/or chemotherapy. This would also benefit the remaining 87% patients, who could be treated more conservatively, sparing them the physical, mental and financial costs of the radical treatment. The long-term goal of this study is not only to find markers for prognosis but also to identify metastasis associated genes. Such knowledge could guide the development of improved therapies. The working hypothesis is that in order to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic alterations in addition to those that led to transformation. In order to isolate genes associated with the suppression of metastasis (whose loss would increase the metastatic potential of the tumor), Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumors with that of the corresponding metastatic lymph node. The primary tumor and metastatic cells were recovered by laser capture microdissection and DNA samples extracted from those cells were used for RDA and for Southern blotting and PCR methods to screen metastasis associated gene sequences (MAGS). Eleven candidate MAGS were recovered that were apparently lost in metastatic cells. One of these, MAGS-IX when used to screen normal, primary and metastatic cell DNA from 3 additional breast carcinomas, was found to be lost in metastatic cell DNA of two of these samples. Thus in 3 out of 4 cases (including the original case used for RDA) MAGS-IX was apparently lost from those primary tumor cells that became metastatic. This makes MAGS-IX a potential candidate for being a metastasis suppressor gene. RH mapping localized MAGS-IX to a 21cR interval between markers, D105539 and D105549, on human chromosome 10, band q21.1. Homology searches revealed it to have 94% sequence identity to a clone on human chromosome 10 (AC022541) but not to any other known gene sequence, suggesting

that this is a novel MAGS. Additional archival cases are being screened to determine if MAGS IX is a reliable marker for identifying breast cancers that are prone to becoming metastatic.

**#221 Shc adaptor proteins in breast cancer prognosis: Novel molecular markers that predict aggressive Stage 1 tumors.** Pamela A. Davol, Robert Bagdasaryan, and A. Raymond Frackelton, Jr. *Roger Williams Medical Center, Providence, RI.*

In a 12-year, retrospective study of clinical outcome (with at least a 5 year follow-up) of breast cancer patients in the Roger Williams Cancer Center database and tumor registry, a 10% mortality associated with disease recurrence was observed in patients diagnosed with Stage 1 breast cancer ( $n = 212$  patients). Accordingly, there is an evident need for molecular markers that may differentiate aggressive, early-stage breast cancers from less invasive lesions and thus guide surgical and adjuvant treatment options. Immunohistochemical staining of phosphorylated Shc (PY-Shc: an activated, adaptor protein that facilitates tyrosine kinase signaling and tumorigenesis) and p66-Shc (a Shc isoform that inhibits this signaling cascade) in 98 archival, formalin-fixed, diagnostic breast tumor biopsies (Stage 0 to Stage 4 patients) demonstrated a positive linear correlation between the ratio of PY-Shc to p66-Shc staining intensity in regard to patient stage ( $r = 0.4$ ;  $p < 0.0001$ ); with high PY-Shc/low p66-Shc corresponding to advanced disease stage at the time of diagnosis. When the PY-Shc to p66-Shc ratio was analyzed in primary tumors from Stage 1 breast cancer patients and then retrospectively compared to patient outcome ( $\geq 5$  yr follow-up), the ratio for randomly selected tumors from patients with no disease recurrence ( $0.66 \pm 0.03$ ;  $n = 30$ ) was significantly lower compared to patients with disease recurrence ( $0.90 \pm 0.07$ ;  $n = 8$ ) ( $p < 0.005$ ). These studies suggest that the PY-Shc to p66-Shc ratio may serve as a viable prognostic marker for identifying aggressive, early stage breast cancers. (Supported by Department of Defense Breast Cancer Grants: BC980415 and DAMD17-99-1-9363)

**#222 Quantitative gene expression of human anterior gradient (hAG-2R) in human breast tumor tissue and its potential as a prognostic indicator in breast cancer.** Monica Madden Reinholz, Stephen J. Ilurria, Patrick C. Roche, and Judith S. Kaur. *Mayo Clinic, Rochester, MN.*

The molecular basis for the observed differences between estrogen receptor-positive (ER+) and ER-negative (ER-) breast tumors remains unclear. The human homologue of the *Xenopus laevis* cement gland gene *Xenopus Anterior Gradient-2* (XAG-2), hAG-2R, was previously found to be co-expressed with ER in breast cancer cell lines. Because ER is probably only one of a set of expressed genes that are responsible for the phenotype of hormone-responsive breast cancer, we examined the gene expression pattern of hAG-2R in different stage breast tumor tissues. In the present study, we used reverse transcription (RT) and fluorescence-based kinetic PCR (Taqman) to determine the mRNA levels of hAG-2R in normal breast tissues, ductal carcinoma in situ (DCIS) tissues, primary breast tumors, and distant breast metastatic tissues. We observed that the average hAG-2R gene expression significantly increased over seven-fold ( $p < 0.0005$ ) in five DCIS tissues and over nine-fold ( $p < 0.0006$ ) in 24 primary breast tumor tissues compared to the average hAG-2R gene expression from 18 normal breast tissues. Eighty percent of the five immunohistochemically detected ER+ DCIS samples overexpressed hAG-2R (overexpression defined as  $> 2$  standard deviations above the mean expression of normal breast tissue). Seventy-four percent of the 19 ER+ primary tumors overexpressed hAG-2R, and only one of the five ER-tumors overexpressed hAG-2R. The average hAG-2R gene expression decreased over 17-fold ( $p < 0.004$ ) in three breast liver metastasis compared to normal breast tissue. In addition, hAG-2R gene expression increased over 45-fold in breast cancer metastatic to bone and was not changed in breast cancer metastatic to ovary compared to normal breast tissue. In a separate panel of 35 node negative breast tumor tissues, 90% of 20 good outcome (indicated by no disease recurrence at five years) tumors overexpressed hAG-2R and 40% of 15 bad outcome (indicated by disease recurrence at less than three years) tumors overexpressed hAG-2R. The average hAG-2R gene expression was four-fold higher in the good outcome tumors compared to the bad outcome tumors. These results demonstrated significant differential gene expression of hAG-2R in different stage breast cancer tissues and the co-expression of hAG-2R and ER in DCIS and primary breast tumor tissues. These results suggest that in addition to ER, hAG-2R may be another gene responsible for the phenotype of hormone-responsive breast cancer. Lastly, hAG-2R gene expression may prove to be a useful prognostic indicator for breast cancer. This work was supported by the DOD grant DAMD-17-00-1-0633, the Mayo Foundation, and the Breast Cancer Research Foundation.

**#223 Nipple fluid basic fibroblast growth factor in breast patients.** Zhi-Ming Shao, Zhen-Zhou Shen, Liping Zhang, Maryam Sartippour, Canhui Liu, He-Jing Wang, Robert Elashoff, Helena Chang, and Mai Nguyen. *University of California, Los Angeles, Los Angeles, CA, and Fudan University, Shanghai, China.*

Purpose: It has been shown that early detection of breast cancer saves lives. Recently, there has been increasing interest in nipple fluid as a potential avenue for breast cancer diagnosis. Experimental Design: In this study, we measured the levels of an angiogenic factor bFGF (basic fibroblast growth factor) in the nipple fluid of healthy subjects as well as patients with benign breast conditions, those at high risk for breast cancer, and patients with ongoing breast cancer. Results:

We found that high risk breasts ( $562 \pm 755$  pg/ml,  $p = 0.009$ ) and cancerous breasts ( $870 \pm 1,848$  pg/ml,  $p = 0.001$ ) produced higher levels of bFGF in nipple fluid in comparison to benign breasts ( $134 \pm 401$  pg/ml). With a cutoff level of 150 pg/ml of bFGF, sensitivity was calculated to be 75%, specificity 84%, and the correct diagnostic rate 82%. Conclusions: We conclude that nipple fluid bFGF may be useful in the diagnosis of breast cancer, and deserves further studies.

**#224 High-level amplification of C-MYC is associated with progression from the *in situ* to the invasive stage of breast carcinomas.** Els C. Robanus-Maandag, Cathy A. J. Bosch, Petra M. Kristel, Augustinus A. M. Hart, Ian F. Faneyte, Petra M. Nederlof, Johannes L. Peterse, and Marc J. van de Vijver. *The Netherlands Cancer Institute, Amsterdam, Netherlands.*

Carcinoma *in situ* of the breast is believed to be a genetically advanced precursor lesion for invasive carcinoma, since *in situ* lesions already demonstrate genomic changes found in invasive lesions. However, no specific genetic alterations have been identified so far that are associated with progression from the *in situ* to the invasive stage. As most invasive carcinomas also contain an *in situ* component, we compared the genetic alterations in the *in situ* and invasive component of the same tumor. Of 12 invasive breast carcinomas, we microdissected the invasive and adjacent *in situ* component, isolated DNA and performed comparative genomic hybridization. In some tumors, we observed a few distinct differences between otherwise identical genome profiles of both components suggesting that the number of genetic alterations involved in breast tumor progression is limited. Further analysis of such a difference in one tumor by fluorescence *in situ* hybridization (FISH) revealed high-level amplification of C-MYC in the invasive component only. To investigate the frequency of this correlation, we identified from a panel of 188 invasive carcinomas 18 cases with C-MYC amplification, 9 of which with an adjacent *in situ* component. Using FISH, more than 5 C-MYC signals per nucleus were found in 7 and C-MYC/CEP8 ratios  $> 4$  were found in 5 of 9 invasive components but not in any associated *in situ* component. With probes of 3 BAC clones derived from chromosome 8q the minimal amplified region in this set of C-MYC-amplified tumors was defined at 8q24.1-8qter. C-MYC amplification was correlated with overexpression of C-MYC and two of its target genes, *TERT* and *FBL*. Thus, high-level C-MYC amplification is the first identified genetic alteration that is strongly associated with progression from the *in situ* to the invasive stage of breast carcinomas.

**#225 Hsp27, angiogenesis and cadherins in human breast cancer biopsy samples.** Mariel A. Fanelli, F. Dario Cuello Carrion, Francisco E. Gago, Olga Tello, and Daniel R. Ciocca. *Institute of Experimental Medicine and Biology of Cuyo (IMBECU), Mendoza, Argentina, and School of Medicine, National University of Cuyo (UNC), Mendoza, Argentina.*

Breast cancer is a heterogeneous disease and the correct identification of the patients who will have a poor prognosis is of clinical value to provide the best treatment options and to plan the follow-up. There are several pathological and molecular prognostic factors and it is clear that the combination of several of them will be necessary to discover the patients with poor prognosis, e.g. those developing distant metastases. In the present study we have evaluated the prognostic significance of hsp27 in the blood vessels of breast cancer patients correlating its expression with that of: a) coagulating factor VIII (FVIII, used to measure angiogenesis), and b) cadherins (E-cadherin and P-cadherin) and beta Catenin. Cadherins are important molecules involved in cell-cell adhesion, some of them have been related with the prognosis of breast cancer. The study involved 113 patients, 76 with a median follow-up of 5 years. The breast cancer biopsy samples were processed for immunohistochemistry. Hsp27 could be detected in the endothelium of small blood vessels as well as in the tumor cells, the number of hsp27-positive vessels was higher in the tumor areas with infiltrating lymphocytes. There was no correlation between the presence of hsp27-positive blood vessels and the amount of blood vessels positive for FVIII, FVIII was a better marker for angiogenesis. The expression of hsp27 in the blood vessels did not correlate with the development of distant metastasis, however, angiogenesis (FVIII) correlated with poor prognosis ( $p(0.02)$ ). Tumors expressing P-cadherin showed more hsp27-negative blood vessels ( $p(0.05)$ ). The presence of P-cadherin (but not E-cadherin) in the membrane of tumor cells was associated with poor prognosis ( $p(0.02)$ ). In tumor cells, hsp27 did not correlate with P-cadherin expression. Beta catenin content did not correlate with P-cadherin expression with disease prognosis. In summary, poor prognosis was seen in patients with P-cadherin expression and with elevated angiogenesis.

**#226 Lipophilin B, lipophilin C, and ECM1, a new member of the uteroglobin family, are overexpressed in endometrial and breast cancer.** Susana Salceda, Anton Nguyen, Carey Drumright, Andrei Munteanu, Melinda Au, Charis Lawrenson, Nam W. Kim, and Roberto A. Macina. *diaDexus, Inc., South San Francisco, CA.*

The members of the uteroglobin family are small, secretory proteins, whose physiological functions remain unclear. Using cDNA databases mining, we have identified a new member of the human uteroglobin family, which we have designated ECM1 (Endometrial Cancer Marker 1). Alignment with data from the Human Genome Project, showed that ECM1 is located on chromosome 11 as has been described for other members of this family. ECM1 protein has 90 amino acids, with a predicted signal peptide in its amino terminal. It shares 61% similarity with lipophilin B, 34% with mammaglobin, and 31% with lipophilin C. Analysis of

**JUNE 15, 2002**  
**Old Dominion University**  
**Constant Hall-Room**  
**9 a.m. - 2 p.m.**

# THE HUMAN

The Challenges and Impact of Human Genome Research

Hosted by  
**ZETA PHI BETA SORORITY, INC.**  
*Beta Theta Zeta Chapter - Norfolk, Virginia*

Funded by  
**Norfolk State University**  
U.S. Office of Education  
Career Development Program in Biotechnology  
Dr. Debabrata Majumdar

Free and Open to the Public

INFORMATION CONFERENCE ON THE HUMAN GENOME PROJECT  
The Challenges and Impact of Human Genome Research

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## CONFERENCE PROGRAM

- 8:30 a.m. Continental Breakfast  
9 a.m. Registration  
9:30 a.m. Greetings
- Purpose of the conference—Stevalynn Adams
- Honorable Yvonne B. Miller—Fifth Senatorial District, Virginia State Senate  
Michelle Ellis Young—President, Beta Theta Zeta Chapter (Norfolk, VA), Zeta Phi Beta Sorority, Inc.
- 10 a.m. Introduction of Keynote Speakers
- The Human Genome Project: From Inception to Present*  
Dr. Bettie J. Graham—Program Director, Division of Extramural Research  
National Human Genome Research Institute (NHGRI)  
National Institutes of Health (NIH), Bethesda, MD
- The Human Genome Project:  
Ethical, Legal and Social Issues of the Human Genome Project*  
Dr. Charmaine Royal—Principal Investigator, GenEthics Unit  
National Human Genome Center, Howard University, Washington, DC
- 11:15 a.m. Break  
11:30 a.m. Panel Discussion
- Moderator:  
Dr. Mark Gray—*Professor of Biology Chemistry and Environmental Science (BCES)*  
Christopher Newport University, Newport News, VA
- Panelists:  
Dr. Mohan Achary—*Cancer Biologist and Assistant Professor*  
Albert Einstein College of Medicine, Bronx, NY  
Rev. Raymond C. Dempsey—*Associate Minister*  
Ebenezer Baptist Church, Virginia Beach, VA  
Amy E. Garber—*Trial Attorney*  
Equal Employment Opportunity Commission's (EEOC)- Norfolk Area Office, Norfolk, VA  
Dr. Debabrata Majumdar—*Professor of Biology*  
Norfolk State University, Norfolk, VA  
Dr. Virginia Proud—*Pediatrician and adult genetic counselor*  
Children's Hospital of the King's Daughters (CHKD), Norfolk, VA  
Dr. Hui Zhao—*Research Associate*  
Albert Einstein College of Medicine, Bronx, NY
- 12:30 p.m. Complimentary Luncheon  
1:15 p.m. Wrap up—What Next?  
1:30 p.m. Presentations, special recognitions and closing

INFORMATION CONFERENCE ON THE HUMAN GENOME PROJECT  
The Challenges and Impact of Human Genome Research

## PANELISTS

### MOHAN ACHARY, PH.D.

*Dr. Mohan Achary* is a cancer biologist and an assistant professor in the Department of Radiation Oncology at Albert Einstein College of Medicine, Bronx, NY. He did his Ph.D. on molecular mechanisms of DNA replication in India. He is presently funded by Radiation Therapy Oncology Group and the Department of Defense Breast Cancer Research Program to develop novel molecular markers for predicting the response of cervical carcinomas to specific types of therapy and for determining which mammary carcinomas will become metastatic. He teaches and supervises medical residents in the departments of Radiation Oncology and Molecular Genetics at Einstein.

### REV. RAYMOND C. DEMPSEY

*Rev. Raymond Dempsey* serves as associate minister at Ebenezer Baptist Church in Virginia Beach. Rev. Dempsey received the Lord Jesus Christ as his personal savior in 1953 at the age of 8 years old and was ordained as a deacon at Community Baptist Church at 18. He was licensed to preach on Aug. 8, 1993.

He attended Marian College, where he obtained a B.S. in business administration in 1975 and completed 42 hours of an MBA program at Wichita State University in 1983. In May 2000 he received a certificate in religious studies from the Evans-Smith Leadership Training Institute, co-sponsored by The Samuel DeWitt Proctor School of Theology of Virginia Union University and the Baptist General Convention of Virginia.

He served in the U.S. Army from 1966-1978.

### AMY E. GARBER

*Amy E. Garber* is a trial attorney with the Equal Employment Opportunity Commission's (EEOC) Norfolk Area Office, Norfolk, VA. She has been with the commission since September 1999 and is one of two attorneys in the Norfolk Area Office. In her capacity as trial attorney she represents the commission when it brings suit against private employers for violations of Title VII of the Civil Rights Act of 1964 (as amended), the Equal Pay Act, the Americans with Disabilities Act and the Age Discrimination in Employment Act.

Prior to joining the commission, Ms. Garber spent almost five years in private practice in Newport News representing employees and employers in employment related matters in federal and state courts and in administrative settings. She is a 1994 graduate of the University of Richmond's T.C. Williams School of Law.

At present she is also functioning half-time as an administrative judge for the commission.

### MARK GRAY, PH.D.

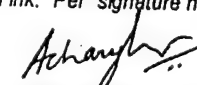
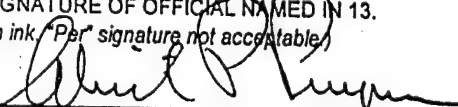
*Dr. Mark Gray*, a professor in the department of biology chemistry and environmental science (BCES) at Christopher Newport University, is an instructor of microbiology, genetics and molecular biology. Dr. Gray earned a Ph.D. in pathology from Thomas Jefferson University in Philadelphia, PA. He continued his studies on the relationship between mechanism of DNA



Department of Health and Human Services  
Public Health Services**Grant Application***Do not exceed 56-character length restrictions, including spaces.*

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)	Date Received	

1. TITLE OF PROJECT <b>Markers of Metastasis in Ductal Mammary Carcinoma</b>			
2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION <input type="checkbox"/> NO <input checked="" type="checkbox"/> YES (If "Yes," state number and title) Number: PA-01-010 Title: Exploratory studies in cancer detection, prognosis and prediction			
3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR		New Investigator <input checked="" type="checkbox"/> No <input type="checkbox"/> Yes	
3a. NAME (Last, first, middle) <b>Achary Patnala Mohanrao</b>		3b. DEGREE(S) <b>Ph.D</b>	
3c. POSITION TITLE <b>Assistant Professor and Cancer Biologist</b>		3d. MAILING ADDRESS (Street, city, state, zip code) <b>Albert Einstein College of Medicine 1300 Morris Park Avenue Bronx, NY 10461</b>	
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT <b>Radiation Oncology</b>			
3f. MAJOR SUBDIVISION <b>Albert Einstein College of Medicine</b>			
3g. TELEPHONE AND FAX (Area code, number and extension) TEL: 718 430 2699 FAX: 718 430 2454			
4. HUMAN SUBJECTS RESEARCH <input type="checkbox"/> No <input checked="" type="checkbox"/> Yes		5. VERTEBRATE ANIMALS <input type="checkbox"/> No <input checked="" type="checkbox"/> Yes	
4a. Research Exempt <input checked="" type="checkbox"/> No <input type="checkbox"/> Yes If "Yes," Exemption No. _____		5a. If "Yes," IACUC approval Date 7/19/00	
4b. Human Subjects Assurance No. FWA00000140		5b. Animal welfare assurance no A3312-01	
4c. NIH-defined Phase III Clinical Trial <input checked="" type="checkbox"/> No <input type="checkbox"/> Yes			
6. DATES OF PROPOSED PERIOD OF SUPPORT (month, day, year—MM/DD/YY) From 04-01-03 Through 03-31-05		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD 7a. Direct Costs (\$) \$100,000	
		7b. Total Costs (\$) 165,660	
		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT 8a. Direct Costs (\$) \$200,000	
		8b. Total Costs (\$) 332,660	
9. APPLICANT ORGANIZATION Name <b>Albert Einstein College of Medicine</b> Address <b>1300 Morris Park Avenue Bronx, NY 10461</b>		10. TYPE OF ORGANIZATION Public: → <input type="checkbox"/> Federal <input type="checkbox"/> State <input type="checkbox"/> Local Private: → <input checked="" type="checkbox"/> Private Nonprofit For-profit: → <input type="checkbox"/> General <input type="checkbox"/> Small Business <input type="checkbox"/> Woman-owned <input type="checkbox"/> Socially and Economically Disadvantaged	
Institutional Profile File Number (if known)		11. ENTITY IDENTIFICATION NUMBER 1131624225A2 DUNS NO. (if available) 071036636 Congressional District 7	
12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE Name <b>Mr. Emanuel Genn</b> Title <b>Associate Dean for Business Affairs</b> Address <b>Albert Einstein College of Medicine 1300 Morris Park Avenue Bronx, NY 10461</b> Tel 718 430 3182 FAX 718 430 8822 E-Mail <b>business @aecom.yu.edu</b>		13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION Name <b>Dominick P. Purpura, M.D.</b> Title <b>Dean</b> Address <b>Albert Einstein College of Medicine 1300 Morris Park Avenue Bronx, NY 10461</b> Tel 718 430 2801 FAX 718 430 8822 E-Mail	
14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.		SIGNATURE OF PI/PPD NAMED IN 3a. (In ink. "Per" signature not acceptable.) 	
		DATE 6/24/02	
15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.		SIGNATURE OF OFFICIAL NAMED IN 13. (In ink. "Per" signature not acceptable.) 	
		DATE 7/1/02	



DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

### Metastasis Associated Molecular Markers in Ductal Mammary Carcinoma

**The long-term objective** is to develop a panel of metastasis associated DNA sequences (MADS) that could be used reliably to identify ductal mammary carcinomas that are prone to developing metastases (Group-I) from those that are not likely to metastasize (Group-II). Consequently attempts will be made in future studies to isolate metastasis suppressor genes, that are existing in the deletion region from which the marker sequence(s) is/are generated by Representational Difference Analysis (RDA). Isolation of novel genes associated with metastasis may identify novel targets for pharmacologic, genetic, or other therapeutic strategies and also should help elucidate the molecular mechanisms and pathways of genes involved in breast metastasis.

**Hypothesis:** To achieve the metastatic state, a primary mammary carcinoma cell must acquire genetic changes in addition to those that led to transformation. This may involve the loss of function of genes due to mutations and/or partial or complete loss of the genes. These deletions may harbor genes that may inhibit metastasis as well as DNA sequences with unrelated functions. Any of these could be used as markers in the prognosis of the metastasis and could also help to identify metastasis associated genes.

**Specific Aim 1:** To isolate a panel of DNA sequences for detecting primary tumors that did develop metastasis (Group-I) and those that did not develop metastasis (Group-II). Previously we have isolated 11 MADS and three of these were shown to be missing in samples of metastasis from additional patients. From subsequent 10 RDA experiments using the 'total probe' method we have identified 4 additional groups of MADS. We propose to screen candidate MADSs step-wise by a quantitative real-time PCR (Q-Rt-PCR) and by fluorescence in situ hybridization (FISH) methods on primary tumors of 40 patients who developed metastases in the lymph nodes (Group-I) and 40 patients who did not develop metastases in the lymph nodes within 5 years of primary tumor detection (Group-II). Tumor cells will be isolated by Laser Capture Microdissection (LCM) for Q-Rt-PCR analysis. We have designed primers for all the MADSs for Q-Rt-PCR and also identified BACs (except one which does not have homology in the gene banks) that contain respective MADSs and also currently using the whole BACs as FISH probes.

**Specific Aim 2:** To determine if the BACs containing MADS have inhibitory effect of metastasis using functional studies. Highly metastatic MDA-MB-435 human mammary carcinoma cells will be transfected with the retrofitted BACs containing MADS of interest. *In vitro* and also an *In vivo* mammary SCID mouse model will be used to determine if the highly metastatic cell line transfected with the BAC/MADS changes to low or non-metastatic phenotype.

### PERFORMANCE SITE(S) (organization, city, state)

Ulmann-1219,  
Albert Einstein College of Medicine,  
1300 Morris Park Avenue, Bronx, NY 10461

**KEY PERSONNEL.** See instructions on Page 11. *Use continuation pages as needed to provide the required information in the format shown below.*

<u>Name</u>	<u>Organization</u>	<u>Role on Project</u>
P. Mohan R. Achary, Ph.D.	Albert Einstein College of Medicine	Principal Investigator
Joan G. Jones, M.D.	Albert Einstein College of Medicine	Pathologist
Harold P. Klinger, MD, PhD.	Albert Einstein College of Medicine	Co-Investigator
Abdissa Negassa, Ph. D.	Albert Einstein College of Medicine	Biostatistician
Robert Russel, Ph.D.	Albert Einstein College of Medicine	Co-Investigator
Hui Zhao, Ph. D.	Albert Einstein College of Medicine	Research Associate

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

## RESEARCH GRANT

### TABLE OF CONTENTS

	Page Numbers
Face Page .....	<u>1</u>
Description, Performance Sites, and Personnel.....	<u>2</u>
Table of Contents.....	<u>3</u>
Narrative Budget Justification.....	<u>4</u>
Biographical Sketch—Principal Investigator/Program Director ( <i>Not to exceed two pages</i> ) .....	<u>5-6</u>
Other Biographical Sketches ( <i>Not to exceed two pages for each</i> ).....	<u>7-16</u>
Resources.....	<u>17</u>
Tables of Users and Utilization.....	
Introduction to Revised Application ( <i>Not to exceed 3 pages</i> ).....	<u>18-20</u>

#### Research Plan

Introduction to Supplemental Application ( <i>Not to exceed 1 page</i> ) .....	
a. Specific Aims.....	<u>21</u>
b. Background and Significance .....	<u>21-23</u>
c. Preliminary Studies/Progress Report .....( <i>Items a-d: not to exceed 25 pages*</i> ) .....	<u>23-32</u>
d. Research Design and Methods .....	<u>32-41</u>
e. Human Subjects.....	<u>41-42</u>
Vertebrate Animals.....	<u>42</u>
Literature Cited.....	<u>43-45</u>
h. Consortium/Contractual Arrangements.....	
Consultants.....	<u>46-52</u>
Checklist.....	<u>53</u>

\*Type density and type size of the entire application must conform to limits provided in instructions on page 6.

Appendix (*Five collated sets. No page numbering necessary for Appendix.*)

Number of publications and manuscripts accepted or submitted for publication (*not to exceed 10*) 5

Other items (*list*): 7

Letters of Institutional Support: 5 (Page 8)

Abstracts: 5 (Page 2)

**X Appendix  
is  
included**

1. AECOM CCI and AIC approval letters (2)

2. Abstracts (2)

3. Publications (5)

4. Critique of the original R-21 grant application (1R21 CA097208-01) ; submitted firstly on 10-1-01 for PA-01-010

5. 5 sets of Color pictures (FISH)

**BUDGET JUSTIFICATION PAGE  
MODULAR RESEARCH GRANT APPLICATION**

**Total direct costs for entire proposed period of support: \$ 200,000**

<u>Initial Period</u>	<u>Second Year</u>
-----------------------	--------------------

\$100,000	\$100,000
-----------	-----------

**Personnel (Effort justification):**

**P. Mohan R. Achary, Ph.D. Principal Investigator (50%):** Dr. Achary's duties will include design, execution, and analysis of the experiments. He will be responsible for the overall project work especially the work related to LCM, FISH and in vivo SCID mouse experiments, preparation of scientific presentations and publications. Dr. Achary will co-ordinate with other investigators of this project and supervise the Research Associate to perform all the required experiments in this project. 50% of his salary is requested.

**Hui Zhao, Ph.D. Research Associate (100%):** Dr. Zhao is highly experienced in all the molecular biology techniques. She will be responsible for microdissection under the direct supervision of the PI, Drs. Klinger and Jones. She will perform Q-Rt-PCR experiments and also will be responsible for ordering the supplies, maintaining the accounting and other records required for the project. Her efforts will be 100% but 30% of her salary is requested.

**Harold P. Klinger, MD, Ph.D. Coinvestigator (10%):** Dr. Klinger will devote 10% of his time for this project. His experience on tumor suppressor genes and his residency training in pathology will be very much useful for this project. He has done microdissection before on ovarian, breast and cervical tumors. He and Dr. Jones will guide the PI and the Research Associate in dissecting the tumor cells by LCM. Additionally he will help in the analysis of the data.

**Joan G. Jones, M.D. Pathologist (5%):** Dr. Jones has experience as a senior surgical pathologist. She will reconfirm type and stage of cancer specimens received from CHTN and other sources and also identify right specimens to be used for screening purposes in this project. She along with Dr. Klinger will guide Drs. Achary and Zhao in the microdissection of tumor cells by LCM. 5% of her salary is requested.

**Abdissa Negassa, Ph.D. Bioistatistician (5%):** Dr. Negassa will be responsible to determine the sample size for screening patient samples and for interpretation of the data generated in our project. 5% salary is requested.

**Robert Russel Ph. D. Coinvestigator:** Dr. Russell is the Scientific Director of Histotechnology and Comparative Pathology Facility in the Department of Pathology at AECOM. He will be responsible for microtomy of ductal mammary carcinoma tissues suitable for LCM and also for FISH studies. He will be also responsible for dissection and processing of the primary tumors, lymph nodes and lung material from the SCID mice to evaluate the metastatic potential of MADS.

**Permanent equipment requested:**

One Dell computer (XPS T550).

## Biographical Sketch of P. Mohan R. Achary, Ph. D.

NAME	POSITION TITLE
Patnala Mohan Rao Achary, Ph.D.	Assistant Professor and Cancer Biologist Dept. of Radiation Oncology

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE/DISTINCTION (if applicable)	YEAR(s)	FIELD OF STUDY
Berhampur University, India	B.Sc. (Hons)	1974	Biology
Berhampur University	M.Sc. First Class (Second Position)	1976	Cytogenetics
Calcutta University	Ph.D.	1987	DNA Replication
NIDDK/NIH, Bethesda, MD.	Post Doc.	1994-'95	Molecular Genetics

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications.

**Professional Experience:**

1994-95: Guest Scientist, NIDDK, NIH, Bethesda, MD.  
 1995-97: Research Associate, Dept. of Radiation Oncology, Albert Einstein College of Med., Bronx, NY.  
 1997-98: Instructor, Dept. of Radiation Oncology, Albert Einstein College of Medicine Bronx, NY.  
 1998- to-date: Cancer Biologist, Dept. of Radiation Oncology, Montefiore Medical Center, Bronx, NY.  
 1999- to-date: Assistant Professor, Dept. of Radiation Oncology, Albert Einstein Coll. of Med., Bronx, NY.

**Awards:**

1. Scientist award by C.S.I.R. (Govt. of India) in 1987 (1987-92).
2. Research Scientist - 'A' award by U.G.C. (Govt. of India) in 1988 (1988-98).
3. Travel Fellowship award by C.S.I.R. to Germany in 1991.
4. Fellow award of Zoological Society of India (FZS) in 1993.
5. Guest Scientist Award by The Foundation for Advanced Education in Sciences, USA, in 1994 (1994-95).
6. IDEA Award (US Army Breast Cancer Research Program) in 1999 (1999-03).
7. I. H. P. Klinger Fund award in 2001 (2001-04).

**Teaching and Research Supervision:**

Teaches medical residency fellows in the Department of Radiation Oncology, MMC/AECOM (1998- to-date);  
 Supervises residents of Radiation Oncology and summer students from different institutions (1997-to-date).

**Membership in Scientific Organizations:**

1. American Association for Cancer Research (Active);
2. Radiation Therapy Oncology Group (Active);
3. American Society for Therapeutic Radiology and Oncology (Associate);
4. Member, RTOG cervical tumor committee
5. American Association for the Advancement of Science (Active);
6. Indian Society for Cell Biology (Life)
7. International Cytogenetics and Genome Society (Life)

**Reviewer of scientific journals and grant Foundations:**

1. Cytogenetic and Genome Research (Editor);
2. Gynecologic Oncology (Reviewer)
3. American Journal of Pathology (Reviewer);
4. Foundation of Ohio Cancer Research Associates (Grant Reviewer)

**Research protocols:**

1. **Principal Investigator:** Isolation of novel genetic lesions in cancer patients by representational difference analysis of archival tissues (i. Molecular markers of metastasis in ductal mammary carcinoma), AECOM CCI# 96-119.
2. **Principal Investigator:** An In Vivo mouse model to study metastasis in breast cancer, AECOM AIC protocol# 000611.
3. **Co-Principal Investigator** (P.I: Dr. Joseph A. Sparano): Molecular Basis for Metastasis and Resistance to Hormonal Therapy in Early Stage Breast Cancer: ER-alpha Mutations, Caveolin-2 Mutations, and Metastasis Associated Genes.

**Research Projects and grant Support:**

1. **Principal Investigator (50%):** "Molecular Markers of Metastasis in Ductal Mammary Carcinoma"; Agency: US Army Breast Cancer Research Program; Type: Idea grant (BC 980731); Duration: August, 1999- July 2002.
2. **Principal Investigator:** Awarded a fellowship by **I. H. P. Klinger Fund (AECOM)** for three years to hire a post-doc. to work on breast and cervical cancer research projects in P. I's (Mohan Achary) lab. Duration: 2001-2004.

**Research Presentations and Publications (Last 3 years):****I. Selected presentations and invited talks in the past 3 years:**

1. P. Mohan R. Achary, R. Khaimov, Z. Fan and B. Vikram, Isolation of Molecular markers of metastasis in mammary carcinoma. **Proceedings of the 89th Annual AACR meeting, New Orleans, LA. March 28th-April 1st, 2001.**
2. P. Mohan R. Achary, B. Mukherjee, Z. Fan, P. Mahadevia and B. Vikram (1999). Search for novel molecular markers to identify patients with breast cancer at high risk for developing metastasis using Representational difference analysis. **90th Annual AACR meeting, Philadelphia. Proceedings of the AACR, April 10-14, 1999, 40: 431.**
3. P. Mohan R. Achary, Gene expression markers in cervical carcinoma, Semi-annual meeting of the **Radiation Therapy Oncology Group, Philadelphia, June 26<sup>th</sup>, 1999.**
4. P. Mohan R. Achary, J. Wayne, H. P. Klinger and B. Vikram (1999). cDNA microarray gene expression analysis of cervical carcinoma cell lines with different degrees of radiosensitivity. **Proceedings of conference on "Prediction of Tumor Response to Therapy " at McGill University, Montreal, Canada, October, 8-9, 1999, Pg. 9.**
5. P. Mohan R. Achary, Molecular markers of breast metastasis and cervical carcinomas, **Faculty meeting of the Department of Radiation Oncology, AECOM/MMC on January 3rd, 2000.**
6. P. Mohan R. Achary, J. Wayne, H. P. Klinger and B. Vikram (2000). Gene expression analysis of cervical carcinoma cell lines with different degrees of radiosensitivity by cDNA microarrays. **Intl. Conference on Translational Research and Strategies in Clinical Radio-Oncology, March 5-8th, 2000. Switzerland. Int. J. Radiation Oncology Biol. Phys. 46:736.**
7. R. Khaimov, E. Gross, J. Liu, J. B. Vikram, and P. Mohan R. Achary, Identification of gene expression profile in a highly metastatic breast carcinoma cell line by cDNA microarray method. **91st Annual AACR meeting, April 1- 5, 2000, Proceedings of the American Association for Cancer Research. 41: 431.**
8. P. Mohan R. Achary, J. Wayne, E. Gross, R. Khaimov, H. P. Klinger and B. Vikram, Gene expression profiles in radioresistant cervix cancer cell lines by cDNA microarray. **91st Annual AACR meeting, April 1- 5, 2000, San Francisco. Proceedings of the American Association for Cancer Research. 41: 709.**
9. P. Mohan R. Achary, Molecular markers of cervical tumor cell lines sensitive to radiation and cisplatin, **The 8<sup>th</sup> meeting of the New York Human Genetics Club, Memorial Sloan-Kettering Cancer Center, October 14th, 2000.**
10. P. Mohan R. Achary, Molecular markers of breast metastasis and cervical carcinomas, **Faculty meeting of the Department of Radiation Oncology, AECOM/MMC on January 6th, 2001.**
11. P. Mohan R. Achary, Molecular markers of breast metastasis and cervical carcinomas, in the **Genetics Research Unit, Calcutta University, India on May 15th, 2001.**
12. Zhao H, Fan Z, Herbst L, Breining D, Jones JG, Mahadevia PS, Klinger HP, Vikram B, P. Mohan R. Achary. A candidate metastasis associated genetic marker for ductal mammary carcinoma. **93<sup>rd</sup> Annual AACR Meeting at San Francisco, California, Proceedings of American Association for Cancer Research, April 6-10, 2002, (Abstract # 220).**
13. P. Mohan R. Achary. Impact of Human Genome Project on Breast cancer research. **Norfolk State Univ., June 15<sup>th</sup>, 2002.**

**II. Selected Publications (last 3 years):**

1. **P. Mohan R. Achary, W. Jaggernauth, E. Gross, A. Alfieri, H. P. Klinger and B. Vikram (2000).** Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression. **Cytogenetics and Cell Genetics 91: 39-43.**
2. **L. H. Herbst, R. Chakrabarty, P. A. Klein, and P. Mohan R. Achary, (2001).** Differential Gene Expression Associated with Tumorigenicity of Cultured Green Turtle FP-Derived Fibroblasts. **Cancer Genetics and Cytogenetics 129: 35-39.**
3. **R. Yuan, S Fan, P. Mohan R. Achary, D. M. Stewart, I. D. Goldberg, and E.M.R. Rosen, (2001)** Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor (HGF/SF) in the setting of DNA damage. **Cancer Research 61: 8022-8031.**

**III. Submitted Manuscripts:**

1. **P. Mohan R. Achary, Zhao H, Fan Z, Herbst L, Mahadevia PS, Jones, J. Klinger HP, Vikram B.** A candidate metastasis associated DNA marker for ductal mammary carcinoma. **Breast Cancer Research.**
2. **B. M. Sood, P. Mohan R. Achary, M. Fazzari and B. Vikram.** The predictive value of gross residual disease after high-dose rate brachytherapy in locally advanced carcinoma of the cervix. **Cancer.**
3. **B. Mukherjee, H. Zhao, B. Parashar, B. M. Sood, P. S. Mahadevia, H. P. Klinger, B. Vikram, P. Mohan R. Achary.** Microsatellite dinucleotide (T-G) repeat: A candidate marker for breast metastasis. **International Journal of Pathology.**

**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed on Form Page 2.  
Photocopy this page or follow this format for each person.

NAME Hui Zhao		POSITION TITLE Research Associate, Dept. of Molecular Genetics	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
China Agricultural University, China	B.Sc.	1993	Horticulture
China Agricultural University, China	M.Sc.	1996	Plant Biotechnology
Chinese Academy of Sciences, Institute of Genetics, China	Ph.D.	1999	Molecular Genetics

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED 2 PAGES.**

**Postdoctoral training:**

1999-00: Post Doc, Molecular Biology Institute, University of California, Los Angeles, California

2000-01: Post Doc, Eye Research Institute, University of North Texas Health Science Center at Fort Worth, Texas

2001.4- to-date: Post Doc, Dept. of Molecular Genetics and Radiation Oncology, Albert Einstein College of Medicine, NY.

**Research Experience:**

Experienced in routine molecular biology techniques, gene transformation, gene knockout, library screening, Representational Difference Analysis (RDA), LCM, cDNA microarray, Genome walking, in vivo mouse tumorigenicity cum metastasis models.

August, 1989- July, 1993:

The influence of defoliating, defruiting and spraying GA3 on the flower formation of Peach trees.

August, 1993 - July, 1996:

The study of the transformation of Bean chitinase gene into Apple Rootstock (*Malus micromalus*)

August, 1996 - September, 1999:

1. Cloning the promoter sequence of ubiquitin gene in Pea infected by Pea seed-borne mosaic virus
2. cDNA gene cloning of wheat chromosome-binding protein
3. Genetic and physiological analysis of Wheat ribonucleases

October, 1999 - September, 2000:

Biochemical and molecular genetic studies of enzymes and proteins involved in the structure and replication of kinetoplast DNA (including protein expression in E.coli, purification and enzyme characterization, site-directed mutagenesis, gene knockout, library screening etc.)

October, 2000 - March, 2001:

Transgenic models of ocular diabetic complications

April, 2001- present:

Isolation and characterization of genes involved in the metastasis of breast carcinoma (by RDA and cDNA microarray)

**Teaching Experience:**

January, 1994-December, 1994: Teaching Assistant for undergraduate students (Biology) in the China Agricultural University.

**Awards and Honors:**

1. 1990-1993 Awarded first-class scholarship for the top 2% of outstanding undergraduates in China Agricultural University (CAU)
2. 1995 Awarded "Excellent Graduate Student" (rendered to the top 5% of outstanding in the graduate school, CAU)
3. 1994-1996 Junior Research Fellowship supported by National High Tech Fund
4. 1997-1999 Senior Research Fellowship by Chinese Academy of Sciences Foundation (K295T-02-02-17)
5. 1999-2000 Postdoctoral Research Fellowship from NIH Grant (GM53254)



6. 2000-2001 Postdoctoral Research Fellowship from NIH/NEI Grant (EY05570)
7. 2001-04 Postdoctoral Research Fellowship by Klingers' Foundation

### Other Support

#### 1. Active :

P.I.: P.M.Achary Ph.D.

Duration: 2001-2003

Agency: Klingers' Foundation, NY.

The long-term goal of this position is to study breast metastasis and radioresistance in cervical carcinomas.

#### 2. Pending:

Title: Markers of Metastasis in Ductal Mammary Carcinoma.

P.I.: P. M. Achary Ph.D.

Duration: 2003-05

Agency: NIH; Type: R-21; Effort: 30%

The long-term goal is to isolate and characterize genetic markers associated with breast metastasis.

### Publications:

1. Zhao H, Song GY, Chen ZH, Zhang GZ (1998) Studies on transformation of bean chitinase gene into apple root stock (*Malus micromalus*). *Acta Laser Biology Sinica*, 7(3): 163-167
2. Zhang J, Zhao H, Wang D. The cloning and expression of protein coding sequence in SMV genome: "Phytopathogenesis Research and Prevention", Selected Works of the Annual Symposium and VI Congress of the Phytopathological Society of China, Edited by Liu Yi 1998, China Agricultural Science and Technology Press, Pgs: 686-687.
3. Zhao H, Wang DW (1999) Recent advances in elucidating the function of the potyviral HC-Pro protein. *Progress in Biotechnology*, 19(6): 53-57
4. Zhang JF, Zhao H, Gui JG, Liu KF & Wang DW (1999) Molecular characterization of a Chinese soybean mosaic virus isolate by RT-PCR, cDNA sequence analysis and direct expression of PCR products in Bacteria. *Acta Botanica Sinica*, 41(9): 932-935
5. Zhao H, Liu KF & Wang DW (2000) A preliminary analysis on a group of low molecular weight RNases in wheat and related species. *Acta Genetica Sinica*, 27(5): 423-426
6. Hines JC, Engel ML, Zhao H, Ray DS (2001) RNA primer removal and gap filling on a model minicircle replication intermediate. *Molecular and Biochemical Parasitology*, 115: 63-67
7. Cammarata PR, Zhao H, Guo Z, Brun AM. Expression of chloride channel and pICln in human lens epithelial cells. Association for Research in Vision and Ophthalmology, 2001 Annual Meeting, Fort Lauderdale, Florida, April 29-May 4, Pg: S875.
8. Zhao H, Fan Z, Mukherjee B, Herbst L, Jones J, Klinger HP, Vikram B and P. Mohan R. Achary, Characterization of Metastasis Associated Gene Sequences in Breast Carcinoma. Fifth Annual Einstein Postdoctoral Symposium, AECOM, December 4th, 2001.
9. Zhao H, Fan Z, Herbst L, Breining D, Jones JG, Mahadevia PS, Klinger HP, Vikram B, P. M. Achary. A candidate metastasis associated marker for ductal mammary carcinoma. 93<sup>rd</sup> Annual AACR Meeting, San Francisco, Proceedings of American Association for Cancer Research, April 6-10, 2002, (Abstract # 220).
10. Achary PM, Zhao H, Fan Z, Herbst L, Mahadevia PS, Klinger HP, Vikram B. A candidate metastasis associated DNA marker for ductal mammary carcinoma. *Breast Cancer Research*, Submitted.
11. Mukherjee B, Zhao H, Parashar B, Sood BM, Mahadevia PS, Klinger HP, Vikram B, Achary PMR (2002) Microsatellite dinucleotide (T-G) repeat: A candidate marker for breast metastasis. *International Journal of Pathology*, submitted.



**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed for Form Page 2.  
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

<b>NAME</b>		<b>POSITION TITLE</b>	
<b>Robert G. Russell</b>		<b>Associate Professor</b>	
<b>EDUCATION/TRAINING</b> (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
<b>INSTITUTION AND LOCATION</b>	<b>DEGREE</b> (if applicable)	<b>YEAR(s)</b>	<b>FIELD OF STUDY</b>
University of Melbourne	BVSc	1963 - 1968	Veterinary Medicine
University of Melbourne	MVSc	1973 - 1975	Virology
University of Saskatchewan	PhD	1975 - 1979	Pathology

**A. POSITIONS AND HONORS.**

1969-1975. Veterinary Medical Office, Veterinary Research Institute, Dept. of Agriculture, Victoria,  
1973-1975 Head, Microbiology Unit for Reproductive Bacteriology.  
1975-1979. Post-doctoral Fellow. Western College of Veterinary Med., Univ. of Saskatchewan  
1979-1984. Assistant Professor, Dept. of Pathology, University of Tennessee.  
1984-1988. Assistant Professor, Division of Animal Medicine, University of Washington  
1988-1998 Research Associate Professor, School of Med., University of Maryland.  
1989-1999 Veterinary Medical Officer, Baltimore Veterans Administration Medical Center.  
1999-present Associate Professor, Department of Pathology, Albert Einstein College of Medicine.

**HONORS:** Gold Medal, Dookie Agricultural College, Australia, 1967  
Phi Zeta, University of Tennessee. 1982.

**BOARD CERTIFICATION:** American College of Veterinary Pathologists

**MEMBERSHIPS:** National Cancer Institute. Mouse Models of Human Cancer Consortium.  
Pathology subcommittee and gastrointestinal organ-site subcommittees. 1999-2001.

**RESEARCH SUPPORT:****ONGOING:**

P30 CA13330-30. I David Goldman (P.I.) 7/1/01-7/31/06

NIH/NCI

\$19 million

Histopathology Budget (year 1): \$113,765

Title: Cancer Center Support Grant

The major goals of the Cancer Center grant is to provide the infrastructure to for support of clinical and basic research programs and shared resources that sustain and benefit the overall research goals of the Comprehensive Cancer Center. Within this program, as director of the Histopathology Facility I administer the histology laboratory and the comparative pathology resource to support cancer studies in genetically engineered mice. No overlap with this grant in which I will provide collaboration for specific cancer-related research. Effort 30%.

1 RO1 HL 689622. R. Nagel (PI) 9/30/01 - 7/31/06

NIH

\$2,974,266

Title: Pleiotropic and epistatic effects in sickle cell anemia

The major goals of this project are to identify genes involved in the vaso-occlusive and vaso-proliferative processes in sickle cell-mediated disease in mouse models, particularly lesions in the retina, choroid and in cerebrovascular complications. Dr Russell provides gross pathology and histopathology support to identify and characterize the pathophysiology in various animal models including necropsy examination, histology, cytology of bone smears and hematocrit analyses. Effort 15%.

**PENDING:**

P.I.: R. Pestell. Anticipated Funding Period: 12/01/02-11/30/03

NIH

Direct Costs First year: \$1,292,988; Total Costs 5 years \$10,273,000.

Title: Transgenic Analysis of Breast Cancer Onset & Progression.

The major goal of this Program grant is to investigate the key molecular events controlling the onset and progression of mammary tumorigenesis. The program involves four projects and four supporting cores. The projects will investigate the role of cyclin D1 in regulating nuclear hormone receptor function, the role of *cav-1*, the role of BRCA1 and the interactions between mammary adipose tissue and tumorigenesis. Dr Russell as Director of the Histopathology Facility will administer the histology laboratory and the comparative pathology resource to support the cancer studies in genetically engineered mice. He will provide pathology expertise for evaluation and classification of the models. No overlap with this grant in which I will provide collaboration for specific cancer-related research. Effort: 15%.

P.I. N. Barzilai, (Program Director) 09/30/02-08/30/07

NIH/ NIA-PPG

\$1,423,350 (First year); (Total Direct Cost)

Title: Excess Nutrients and the Metabolic Syndrome of Aging.

We hypothesize that aging is characterized by a decline in hypothalamic function leading to a relative or absolute increase in energy intake, Increased fat mass and impaired regulation of fat distribution. While the effects of increase of nutrients leads to changes in adiposity and its distribution, we will attempt to assess the impact of each of these individually with aging. Dr. Russell as Director of the Histopathology Facility will on these studies. Effort 5%.

## PUBLICATIONS. (selected).

- Russell, R.G., Kiehlbauch, J.A., Sarmiento, J.I., Panigrahi, P., Blake, D.C. Jr. and Haberberger, R. Ribosomal RNA patterns identify additional strains of *Campylobacter jejuni* and *C. coli* among isolates serotyped by heat-stable and heat-labile antigens. (1994). Lab. Anim. Sci. 44: 579-583.
- Barnes G.M., Delaney, P.A., Gheorghiu, I., Mandava, S., Russell, R.G., Kahn, R and Mackenzie, C. F. (1997). Respiratory impedances and acinar gas transfer in a canine model for emphysema. J. Appl. Physiol. 83:179-188.
- Furth, P.A., Bar-Peled, U., Li, M., Lewis, A., Lauceric, R., Jager, R., Weiher, H and Russell, R.G. (1999). Loss of anti-mitotic effects of *Bcl-2* with retention of anti-mitotic activity during tumor progression in a mouse model. Oncogene 18: 6589-96.
- Hondalus, M.K., Bardarov, S., Russell, R.G., Chan, J., Jacobs, W.R., and Bloom, B.R. (2000). Attenuation and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. Infect. Immun. 68: 2888-2898.
- DeSousa, A., Mazzaccaro, R.J., Russell, R.G., Lee, F.K., Turner, O.C., Hong, S., Van Kaer, L. and Bloom, B.R. (2000). Relative contributions of distinct MHC class I-dependant cell populations in protection to tuberculosis in mice. PNAS. 97: 4204-4208.
- Puech, A., Saint-Jore, B., Merscher, S., Russell, R.G., Cherif, D., Sirotkin, H., Xu, H., Factor, S., Kucherlapati, R., Skoultschi, A.I. (2000). Normal Cardiovascular Development in Mice Deficient for 550kb of the Velo cardio facial/diGeorge Syndrome Region. PNAS. 97:4204-4208.
- Lu, Y., Lian, H., Sharma, P., Schreiber-Agus, N., Russell, R.G., Chin, L., van der Horst, G. and Bregman, B.B. (2001). Disruption of the Cockayne Syndrome B gene impairs spontaneous tumorigenesis in cancer-predisposed *Ink4/ARF* knockout mice. Molec. Cell. Biol. 21:1810-1818.
- Merscher, S., Funke, B., Epstein, J.A., Heyer, J., Puech, A., Lu, M.M., Xavier, R.J., Demay, M.B. Russell, R.G., Factor, S., Tokooya, K., St. Jore, B., Lopez, M., Pandita, R.J., Lia, M., Carrion, D., Xu, H., Schorle, H., Kobler, J.B., Scrambler, P., Wynshaw-Boris, A., Skoultschi, A., Morrow, B., and Kucherlapati, R. (2001). *TBX1* is responsible for cardiovascular defects in Velo-Cardio-Facial/DiGeorge Syndrome. Cell 104:619-629.
- Lin E.Y. Nguyen, A.V., Russell, R.G., and Pollard J.W. (2001). CSF-1 promotes the progression and metastasis but not growth of primary tumors in a mouse strain with spontaneous mammary cancer. J. Exp. Med. 193:727-739.
- Ryan, G.R., Dai, X-U., Dominguez, M., Tong, W., Chuan, F., M. Chisholm, O., Russell, R.G., Pollard, J., and Stanley, E.R. (2001). Rescue of the Colony-stimulating factor 1 (CSF-1)-nullizygous mouse (*Csf1<sup>op</sup>/Csf1<sup>op</sup>*) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. Blood 98:74 - 84.
- Zhao, R., Russell, R.G., Wang, Y., Liu, L., Gao, F., Kneitz, B., Edelmann, W. and Goldman, I.D. (2001). Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs. J. Biol. Chem. 276:10224-10228.
- Wang Y. Zhao R. Russell RG. Goldman ID. (2001). Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. Biochimica et Biophysica Acta. 1513:49-54.
- Fan, Y., Sirotkin, A., Russell, R.G., Ayala, J., and Skoultschi, A.I. (2001). Individual somatic h1 subtypes are dispensable for mouse development even in mice lacking the h1<sup>o</sup> replacement subtype. Molec. Cell. Biol. 21: 7933-7943.
- Razani, B., Engelman, J.A., Wang, X.B., Schubert, W., Zhang, X.L., Marks CB. Macaluso, F., Russell, R.G., Li, M., Pestell, R.G., DiVizio, D., Hou, H. Jr., Kneitz, B., Lagaud, G., Christ, G.J., Edelmann, W. and Lisanti, M.P. (2001). Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities.
- Xu-Ming, D., Ryan, G.D., Hapel, A.J., dominquez, M.G., Russell, R.G., Kapp, S., Sylvestre, V. and Stanley, E.R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood 99:111-120.

**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed for Form Page 2.

Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME
 POSITION TITLE

Harold P. Klinger, M.D. Ph.D.
 Professor of Molecular Genetics and Pediatrics

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Harvard College, Cambridge, Mass.	B.A.	1952	Biology
University of Basel, School of Medicine, Basel, Switzerland	M.D.	1959	Medicine
University of Basel, Division of Natural Sciences, Basel, Switzerland	Ph.D.	1963	Zoology

**RESEARCH AND PROFESSIONAL EXPERIENCE**

- 1943 - 1952 Junior Research Assistant, Spinal cord compression studies, Department of Neurosurgery, Brooklyn Jewish and Flower Fifth Ave. Hospitals, New York
- 1948 - 1952 Undergraduate Research in Cytology, Department of Histology, Harvard Biological Laboratories
- 1956 - 1957 Demonstrator in Anatomy, Department of Anatomy, Basel, Switzerland
- 1957 - 1959 Resident in Pathology, Department of Pathology, University of Basel, Switzerland and Research Fellow 'Roche' Study Foundation and Swiss National Cancer Institute
- 1960 - 1963 Director of the Cytogenetic Research Unit of the Dept. of Anatomy of the Univer. of Basel, Switzerland
- 1962 - Founder and Chief Editor of the journal **CYTOGENETICS AND CELL GENETICS** (presently, **CYTOGENETIC AND GENOME RESEARCH**) published by S. Karger, AG, Basel and New York
- 1963 - Consultant and member Scientific Advisory Committee of the Ford Foundation
- 1963 - 1967 Assistant Professor of Anatomy and Genetics and Cytogeneticist at the Human Heredity Clinic, Albert Einstein College of Medicine, New York
- 1965 - 1974 Research Career Development Award, NIH, Division of General Medical Sciences
- 1965 - Consultant to the World Health Organization
- 1965 - 1995 Editor of several reports of the International Committee for Human Cytogenetic Nomenclature and ten International Human Gene Mapping Workshop reports
- 1967 - 1970 Member of National Institutes of Health Human Embryology and Development Study Section
- 1967 - 1972 Associate Professor of Genetics, Albert Einstein College of Medicine, New York
- 1968 - Co-director of Genetic Counseling Clinic of Albert Einstein College of Medicine
- 1971 - Member Advisory Committee, The Population Council, Rockefeller University, New York
- 1972 - Professor of Molecular Genetics and Pediatrics, Albert Einstein College of Medicine
- 1975 - 1985 Program Director, NIH sponsored Research Training Program in Medical Genetics
- 1987 - 1995 Elected Executive Committee Member, International Workshops on Human Gene Mapping presently a part of the Human Genome Organization (HUGO).
- 2001 - Founder of **INTERNATIONAL CYTOGENETICS AND GENOME SOCIETY** (Co-Founder: Prof. M. Schmidt; Supported by S. Karger, AG, Basel and New York).

**Research Projects and grant Support:**

1. Co Investigator: "Molecular Markers of Metastasis in Ductal Mammary Carcinoma"; Agency: US Army Breast Cancer Research Program; Type: Idea grant (BC 980731); Duration: August, 1999- July 2002. Effort=20%
2. Principal Investigator 50% effort "Support of Editorial Office - *Cytogenetic and Genome Research* Published by S. Karger, Basel Switzerland and New York. S. Karger AG Grant College No. 9526 - 9032 January 1, 2002 - Indefinite termination Annual direct costs \$145,000

**Selected Publications and recent presentations**

- KLINGER, H.P. and SCHWARZACHER, H.G.: Sex chromatin in polyploid nuclei of human amnion epithelium. *Nature* **181**: 1150-1151 (1958).
- HAMERTON, J.L., FRACCARO, M., DeCARLI, L., NUZZO, F., KLINGER, H.P., HULLIGER, L., TAYLOR, A. and LANG, E.M.: Somatic chromosomes of the gorilla. *Nature* **192**: 225-228 (1961)
- CORI, C., GLUECKSON-WAELSCH, S., KLINGER, H. P., PICK, L., SCHLAGMAN, S. L., TEICHER, L. Z. and WANG-CHANG, H.-F.: Complementation of gene deletions by cell hybridization. *Proc. nat. Acad. Sci., USA* **78**: 479-483 (1981).
- EUN, C.K., PAIK, S.G., GOLDWASSER, P., SHIN, S. and KLINGER, H.P.: Immunochemical identification of the chick *HPRT* gene transferred from chick erythrocytes to mammalian somatic cells. *Cytogenet. Cell Genet.*, **29**: 116-121 (1981).
- KLINGER, H.P. and SHOWS, T.B.: Suppression of tumorigenicity in somatic cell hybrids. II. Human chromosomes implicated as suppressors of tumorigenicity in hybrids with Chinese hamster ovary cells. *J. Nat. Cancer Inst.* **71**: 559-569 (1983).
- O'HARA, B.M., KLINGER, H.P., CURRAN, T., ZHANG, Y-D., and BLAIR, D.G.: Levels of FOS, ETS2 and MYB protooncogene RNA's correlate with segregation of chromosome 11 of the normal cell and with the suppression of tumorigenicity in human cell hybrids. *Mol. Cell. Biol.* **7**: 2941-2946 (1987).
- O'HARA, B., JOHANN, S.V., KLINGER, H.P., BLAIR, D.G., RUBINSON, H., DUNN, K.J., SASS, P., VITEK, S.M., and ROBINS, T.: Characterization of a human gene conferring sensitivity to infection by gibbon ape leukemia virus. *Cell Growth & Differentiation* **1**:119-127 (1990).
- KAEHLING, M., EDDY, R., SHOWS, T.B., COPELAND, N.G., GILBERT, D.J., JENKINS, N.A., KLINGER, H.P., and O'HARA B.: Localization of the human gene allowing infection by gibbon ape leukemia virus to human chromosome region 2q11-q14 and to the homologous region on mouse chromosome 2. *J. Virol.* **65**:1743-1747 (1991).
- KAEHLING, M., BURK, R.D., ATKIN, N.B., JOHNSON, A.B., and KLINGER, H.P.: Loss of heterozygosity on chromosome 17p and mutant p53 in HPV-negative cervical carcinomas. *Lancet* **340**: 140-142 (1992).
- CRUCIANI, R.A., DVORKIN, B., KLINGER, H.P., and MAKMAN, M.H.: A novel neuronal receptor selective for opioid alkaloids. *Brain Research* **667**: 229-237 (1994).
- MULLOKANDOV M., KHOLLODILOV N.G., ATKIN N.B., BURK R.D., JOHNSON A.B. and KLINGER H.P.: Genomic alterations in cervical carcinoma: Losses of chromosome heterozygosity and HPV tumor status. *Cancer Res.* **56**: 197-205 (1996).
- BOLDOG, F., GEMMILL, R.M., WEST, J., ROBINSON, M., ROBINSON L., LI, E., ROCHE, J., TODD, S., WAGGONER, B., LUNDSTROM, R., JACOBSON, J., MULLOKANDOV, M., KLINGER, H.P. and DRABKIN, H.A.: Chromosome 3p13 Homozygous deletions and sequence analysis of FRA3B. *Human Molecular Genetics* **6**: 193-203 (1997).
- P. MOHAN R. ACHARY, J. WAYNE, E. GROSS, R. KHAIMOV, H. P. KLINGER AND B. VIKRAM, Gene expression profiles in radioresistant cervix cancer cell lines by cDNA microarray. 91st Annual AACR meeting, April 1- 5, 2000, San Francisco. *Proceedings of the American Association for Cancer Research.* **41**: 709.
- P. Mohan R. Achary, J. Wayne, H. P. Klinger and B. Vikram (2000). Gene expression analysis of cervical carcinoma cell lines with different degrees of radiosensitivity by cDNA microarrays. *Intl. Conference on Translational Research and Strategies in Clinical Radio-Oncology*, March 5-8th, 2000. Switzerland. *Int. J. Radiation Oncology Biol. Phys.* **46**:736.
- P. MOHAN R. ACHARY, W. JAGGERNAUTH, E. GROSS, A. ALFIERI, H. P. KLINGER AND B. VIKRAM. Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression. *Cytogenetics and Cell Genetics* **91**: 39-43. (2000).
- I. CASS, MULLOKANDOV M, FIELDS AL, KUO DY, ANDERSON PS, RUNOWICZ CD, KLINGER HP: Allelic losses and microsatellite instability in ovarian carcinoma. *Cancer Res.* (In press, 2002)
- P. MOHAN R. ACHARY, ZHAO H, FAN Z, HERBST L, MAHADEVIA PS, JONES, J. KLINGER HP, VIKRAM B. A candidate metastasis associated DNA marker for ductal mammary carcinoma. *Breast Cancer Research* (Submitted).
- B. Mukherjee, H. Zhao, B. Parashar, B. M. Sood, P. S. Mahadevia, H. P. Klinger, B. Vikram, P. Mohan R. Achary. Microsatellite dinucleotide (T-G) repeat: A candidate marker for breast metastasis. *International Journal of Pathology* (Submitted).

**BIOGRAPHICAL SKETCH**

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Follow the sample format on preceding page for each person. **DO NOT EXCEED TWO PAGES.**

NAME		POSITION TITLE	
JOAN G. JONES, M.D.		DEPUTY DIRECTOR, ANATOMIC PATHOLOGY	
EDUCATION <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
POMONA COLLEGE	B.A.	1969	FRENCH LITERATURE
CLAREMONT GRADUATE SCHOOL	M.A.	1971	EDUCATION
OCCIDENTAL COLLEGE		1972-74	PSYCHOLOGY
COLUMBIA UNIV. SCHOOL OF GENERAL STUDIES		1978-80	PREMED
ALBERT EINSTEIN COLLEGE OF MEDICINE	M.D.	1984	MEDICINE

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **DO NOT EXCEED TWO PAGES.**

**Postgraduate Training: (selected)**

1984-88 Resident, Anatomic Pathology, Albert Einstein College of Medicine  
 1986-88 Chief Resident, Department of Pathology, AECOM

**Professional Employment:**

1988 Assistant Professor, Department of Pathology, AECOM  
 1993 Associate Professor, Department of Pathology, AECOM  
 1997 Associate Professor, Department of OB/GYN & Women's Health  
 1998 Professor, Department of Pathology, AECOM

**Hospital Appointments:**

1988 Attending, Surgical Pathology, Bronx Municipal Hospital Center  
 1990 Attending, Surgical Pathology, WHAECOM  
 1994 Co-Director, Anatomic Pathology, WHAECOM  
 1997 Deputy Director, Anatomic Pathology, WHAECOM

**Awards and Honors:**

Alpha Omega Alpha  
 Samuel M. Rosen Outstanding Teacher Award  
 President, Leo M. Davidoff Society (1995-98)

**Grant Support:**

Title: Molecular markers of metastasis in ductal mammary carcinoma. Supporting Agency: U.S. Army. Investigators and % effort: P. M. Achary, P.I. (100%), B. Mukhopadhyay, CO/P.I. (20%), H. P. Klinger, CO/P.I. (10%), B. Vikram, CO/P.I. (5%), J. Jones, Investigator (10%), C. Fann, Statistician (2%). 1999-2002.

Title: FISH & CHIPS: Single cell expression of cancer genes. Supporting Agency: NIH. Investigators and % effort: R.H. Singer P.I. (10%), G. Childs (5%), J. Condeelis (5%), W. Edelmann (5%), B. Wenig (5%), L. Augenlicht (5%), J. G. Jones (25% - yr 4), 7/1/99-6/30/03.



**Title:** Novel imaging methods for gene discovery in cancer. Supporting Agency: NIH. Investigators and % effort: J. Condeelis P.I. (20%), J. E. Segall Co PI (5%), J. Pollard Co PI (5%), J. G. Jones Co PI (10% effort – years 2-4), 7/1/00-6/30/04.

**Publications: (Selected)**

- (1) Dutcher JP, Benchabbat A, Jones JG, Wiernik PH. Unique dermatological complication of rhM-CSF treatment. Leukemia and lymphoma (15):347-349, 1994.
- (2) Lerner SE, Jones JG, Fleischmann J. Management of recurrent penile cancer following partial of total penectomy. Urologic Clinics of North America Vol 21 (4), Guest ed. Martin L. Resnick, Philadelphia: 729-737, 1994.
- (3) Smith HO, Kuo DYS, Anderson PS, Goldberg GL, DeVictoria CL, Babcock CA, Jones JG, Runowicz CD, Stanley ER, Pollard JW. The role of colony stimulating factor-1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. Clinical Cancer Research 1(3):313-325, 1995.
- (4) Tobias DH, Smith HO, Jones JG, Anderson P, Runowicz CD, Goldberg GL. Cutaneous metastases from squamous cell carcinoma of the vulva. Eur J Gynaec Oncol 16(5):382-386, 1995.
- (5) Segall JE, Tyerech S, Boselli L, Masseling S, Heift J, Chan A, Jones JG, Condeelis J. EGF stimulated lamellpod extension in metastatic mammary adenocarcinoma cells by an actin-dependint mechanism. Clinical and Experimental Metastasis 14(1):61-72, 1996.
- (6) Comerici JT, Jones JG, Fields AL, Runowicz CD, Goldberg GL. Squamous cell carcinoma in a mature cystic teratoma in a young woman: a diagnostic and management dilemma. Eur J Gynaec Oncol 17(6):501-503, 1996.
- (7) Edmonds BT, Wyckoff J, Yeung Y-G, Wang Y, Stanley ER, Jones JG, Segall J, Condeelis J. Elongation factor-1a is an over expressed actin binding protein in a metastatic rat mammary adenocarcinoma. J Cell Science 109:2705-2714, 1996.
- (8) Kuo D Y-S, Mallick S, Shen H-J, DeVictoria C, Jones JG, Fields AL, Goldberg GL, Runowicz CD, Horwitz SB. Analysis of MDR1 expression in normal and malignant endometrium by reverse transcription-polymerase chain reaction and immunohistochemistry. Clin Cancer Res 2:1981-1992, 1996.
- (9) Mukherjee T, Abadi M, Tsai T, Copperman AB, Jones JG, Levгур M. The effect of prolonged GnRH agonist administration on uterine leiomyomata histopathology. J Gynecol Surg 12:251-256, 1996.
- (10) Fehmian C, Jones, JG, Kress Y, Abadi M. Adenosarcoma of the uterus with extensive smooth muscle differentiation: Ultrastructural study and review of the literature. Ultrastructural Pathology 21:73-79, 1997.
- (11) Kuo D Y-S, Jones JG, Fields AL, Runowicz CD, Goldberg GL. Endometrioid adenocarcinoma of the ovary and long term tamoxifen therapy: A coincidence or a cause for concern? Eur J Gyn Oncol 18:457-460, 1997.
- (12) Farina K, Wyckoff J, Rivera J, Lee H, Segall J, Condeelis J, Jones JG. Cell motility of tumor cells visualized in a living intact primary tumor using green fluorescent protein. Clin Cancer Res 58:2528-2532, 1998.
- (13) Shestakova E, Wyckoff J, Jones JG, Singer RH, and Condeelis J. Correlation of  $\beta$ -actin messenger RNA localization with metastatic potential in rat adenocarcinoma cell lines. Cancer Research 59: 1202-1205, 1999.
- (14) Anderson P, Smith H, Jones JG, Goldberg G, Fields A, Runowicz C and Pollard W. Colony-stimulating factor-1 and its receptor do not have a role in the pathogenesis of uterine sarcomas. Gyn Oncol 72 (2): 202-207, 1999.
- (15) Badve S, Fehmian C, Cass I, Goldberg GL, Jones JG. Malignant brenner tumor mimicking a primary squamous cell carcinoma of the cervix. Gyn Oncol. 74 (3): 487-490, 1999.
- (16) Sheshtakova EA, Wyckoff J, Jones J, Singer RH, Condeelis J. Correlation of B-actin messenger RNA localization with metastatic potential in rat adenocarcinoma cell lines. Cancer Res 59: 1202-1205, 1999.
- (17) Wyckoff J, Condeelis J, Jones J and Segall J. In vivo analysis of intravasation as the rate limiting step in metastasis. Cancer Res 60: 2504-2511, 2000.

**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed on Form Page 2.

Photocopy this page or follow this format for each person.

NAME	POSITION TITLE		
Abdissa Negassa	Assistant Professor, Dept. Epidemiology & Social Medicine		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Addis Ababa University, Addis Ababa, Ethiopia	B.S.	1985	Statistics
McGill University, Montreal, Canada	M.S.	1991	Epidem./Biostatistics
McGill University, Montreal, Canada	Ph.D.	1996	Epidem./Biostatistics
RESEARCH AND RESEARCH RESULTS			

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

**PROFESSIONAL EXPERIENCE:**

1995-1997 Biostatistical Consultant, Dental Clinic, Montreal Children's Hospital, McGill University, Montreal, Canada  
 1996-1997 Instructor, Analysis of Multivariable Data, Dept. Epidemiology/Biostatistics, McGill University, Montreal, Canada  
 1998-2000 Asst. Professor, Dept. Clinical Epidemiology & Biostatistics, McMaster University, Hamilton, Canada  
 1998-2000 Biostatistician, Dept. of Medicine, McMaster University, Hamilton Canada  
 2000-pres. Asst. Professor, Dept. Epidemiology & Social Medicine, Albert Einstein College of Medicine, Bronx, NY

**PROFESSIONAL ASSOCIATIONS:**

2002 American Statistical Association

**ACADEMIC AWARDS:**

1989-1991 CIDA Scholarship  
 1992-1996 Research Trainee, Heart and Stroke Foundation of Canada  
 1997 Bursary, International Agency for Research on Cancer, World Health Organization

**Other Support****ACTIVE**

P30 13330-29 (Goldman) 07/01/01-03/31/06 25%  
 NIH \$3,073,744  
 Cancer Center Core Grant  
 The goal of the Center is to foster basic, clinical, population-based and translational research that addresses all aspects of the cancer problem.

K30 HL0411003 (Marantz) 9/1/99 - 8/31/04 5%  
 NIH/NHLBI \$1,121,736  
 Goals: To train clinicians in a program which combines a didactic course of study in statistics, epidemiology, computing, study design, research ethics, and scientific communications (leading to a new degree, Masters of Science in Clinical Research Methods) and mentored original research project(leading to master's thesis). The program's mission is to prepare these scholars for successful careers in clinical research, across the entire spectrum of activities defined by that term.

9526-3878 (Rohan) 07/01/01 06/30/06 10%  
 NIH/NCI \$ 658, 457  
 Fat Reduction, HRT Use and Benign Breast Disease Risk

This study is designed to test the following hypothesis: 1) Adoption of low-fat dietary pattern is associated with reduced risk of proliferative forms of benign breast disease (BBD); 2) Calcium/Vitamin D (CaD) supplementation is associated with reduced risk of BBD; and 3) Use of estrogen replacement therapy (ERT) or combined estrogen/progestogen therapy (PERT) is associated with increased risk of BBD. The study proposes to use the Women's Health Initiative (WHI) randomized clinical trial to test these hypotheses.

R000 (Rohan) 4/1/02-3/31/07 10%  
 \$697,233

P53 in BBD and Breast Cancer Risk: A Multicenter Cohort



The goal of this study is to estimate the risk of breast cancer in association with *p53* mutation and/or *p53* protein accumulation in benign breast tissue obtained from 1,005 cases and 1,615 controls prior to the occurrence of breast cancer (for the cases) and at a comparable time (for the controls). This study will be undertaken as a nested case-control study within a large multi-center (international) cohort of 25,843 women who were biopsied for benign breast disease and who have been followed-up to determine the subsequent occurrence of breast cancer.

**PENDING**

R0000	(Fang)	01/01/02 - 31/12/04	15%
Agency for Health Care Research & Quality		\$113,418	

## Community Disparities in Access to Revascularization Procedures in New York City

The goals of this study are to: 1) Determine community-level factors associated with cardiac revascularization; 2) To quantify coronary heart disease mortality in different communities; and 3) To compare trends of cardiac procedure and coronary heart disease outcomes among different communities over three decades. The study will use administrative and census data to achieve these goals.

R000	(Smoller)	12/01/2001-05/31/2006	10%
NIH	\$349,682		

The goal of the proposed study is to determine whether blood-based biomarkers and polymorphism in candidate genes related to systemic inflammation, hemostasis, and blood pressure regulation may be important stroke risk factors among older women. The study will be conducted as a population based nested case-control study of 1100 stroke cases and 1100 matched controls among participants in the Observational Study component of the Women's Health Initiative (WHI-OS).

R000	(Kaufman)	7/1/02-6/1/04	5%
NIH/NCI		\$250,000	

Enhancing Tumor Vaccines with Co-stimulatory Molecules The focus of this grant application is to improve vaccination strategies by immunizing with novel vaccines expressing co-stimulatory molecules, thus increasing the likelihood of activating tumor-specific T-cells.

R000 (Kadish)		10%
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Surrogate biomarkers for efficacy of HSP-7 vaccine

The study hypothesizes that HspE7 vaccination will result in regression of CIN in vaccinated women and enhance CMI response to the E7 protein as well as other HPV proteins (E4, E6) and unrelated antigens (e.g., candida and tetanus).

**OVERLAP:** None.

**PUBLICATIONS:**

Schlecht N, Franco LE, Pintos J, Negassa A, Kowalski LP, et al. Interaction between tobacco and alcohol consumption on the risk of cancer of the upper aero-digestive tract in Brazil. *Am J Epidemiol* 1999; **150**:1129-1137.

Hanley AJ, Negassa A, Edwards DM. GEE Analysis of negatively correlated binary response: A caution. *Stat in Med* 2000; **19**:715-722.

Capes ES, Gerstein CH, Negassa A, Yusuf S. Enalapril prevents clinical proteinuria in patients with low ejection fraction. *Diabetic Care* 2000; **23**:377-380.

Negassa A, Ciampi A, Abrahamowicz M, Shapiro S, Boivin JF. Validation of tree structured prediction for censored survival data: Its application to prognostic classification. *The Journal of Statistical Computation and Simulation* 2000; **67**:289-317.

Suskin N, Sheth T, Negassa A, Yusuf S. Relationship of current and past smoking to mortality and morbidity in patients with left ventricular dysfunction. *J Am Coll Cardiol* 2001; **37**: 1677 - 82.

Ounpuu S, Negassa A, Yusuf S for The INTER-HEART investigators. INTER-HEART: A Global study of risk factors for acute myocardial infarction - rational and design. *Am Heart J* 2001; **141**: 711-21.

Salman H., Perez A., Sparano J. A., Ratech H., Negassa A, Hopkins U and Wiernik P. H. Phase II trial of infusional cyclophosphamide, idarubicin, and etoposide in poor-prognosis non-Hodgkin's lymphoma. *American Journal of Oncology* (in press).

Yusuf S, Negassa A. Choice of clinical outcomes in randomized clinical trials of heart failure therapies: Disease specific or overall outcomes? *Am Heart J* (in press).

Salman H., Cynamon J., Jagust M., Bakal C., Rozenblit A., Kaleya R., Negassa A, Wadler S. Randomized phase II trial of embolization therapy versus chemoembolization therapy in previously treated patients with colorectal carcinoma metastatic to the liver (under review).

Hanley AJ, Negassa A, Edwards DM, Forrester JE. Statistical analysis using Generalized Estimating Equations (GEE): An orientation (under review).

McKelvie R. S., White M., Rouleau J-L., Maggioni A. P., Negassa A, Young J. B., Held P. and Yusuf S. Single versus Double versus Triple Therapy to Block Ventricular Remodeling in Patients with Congestive Heart Failure (under review).

## RESOURCES

**FACILITIES:** Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services.

### Laboratory:

The P.I's laboratory (538 Sq. ft.) is situated in the room # 704A of Forchheimer building at Albert Einstein College of Medicine (AECOM). It has all the equipment and bench space required for conducting the experiments proposed in this project. We have access to use the instruments in the AECOM central facility, Cancer Center and also the laboratories of Dr. H. P. Klinger and Dr. Jack Lenz (Dept. of Molecular Genetics), and Dr. Russel (Dept. of Pathology).

### Clinical:

Pathology departments of Weiler and Jacobi hospitals of Einstein College are within the campus.

### Computers:

We have one PC (Pentium II) and a Dell computer (Pentium III) with laser printer exclusively for the PI's lab. They are connected with network for communications and various databases and libraries.

### Office:

The P. I.'s research office with telephone and fax facilities is located in room 704C. Secretarial support is available in the Department of Radiation Oncology.

### Other facilities:

1. Core facilities: We have cDNA microarray facility with Genepix scanner, Sequencing and **Oligonucleotide synthesis** facilities in the Department of Molecular Genetics, AECOM.
2. **Laser Capture Microdissection facility:** We have our own laser capture microdissection (Arcturus Co.) facility for the isolation of cells from tissue sections.
3. **Cytogenetics and FISH facility:** We have access to the facility. Dr. Linda Cannizzaro is the Director and she collaborates in our projects.
4. **Microtomy and histopathology facility:** Dr. Russell is the Director and he collaborates with our projects.
5. **Animal facility:** Dr. Herbst is the Associate Director and also collaborates in our projects.
6. The AECOM's engineering and electronic repairing services are available.

## MAJOR EQUIPMENT:

Real-time SmartCycler PCR equipment (Cepheid), Perkin Elmer PCR thermocycler for 500uli tubes, Appligene/Oncor PCR thermocycler with provisions to use with 200uli, 500uli tubes and also slides, Zeiss phase contrast microscope, Nikon microscope fitted with a microdissector, Milli Q water facility, water shaking baths, three incubators, CO2 incubator, laminar flow, electrophoresis apparatus, UV transilluminator, freezer minus 70C, freezers minus 20C (one small and one very large), vacuum dessicator, liquid nitrogen containers, Ice machine, etc. The instruments like centrifuges, ultracentrifuges, spectrophotometer, UV cross linker, gel documentation system, cold room, dry ice etc. are available for sharing with the Departments of Molecular Genetics and Pathology.



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health  
National Cancer Institute  
Bethesda, Maryland 20892

6130 Executive Blvd, Suite 6035A

March 25, 2002

Dr. Patnala M. Achary  
Albert Einstein College of Med  
Dept of Radiation Oncology  
1300 Morris Park Avenue  
Bronx, NY 10461

Reference: 1 R21 CA097208-01

Dear Dr. Achary:

Enclosed is a copy of the scientific evaluation (Summary Statement) for your application referenced above. Your application will be reviewed by the National Cancer Advisory Board (NCAB) at its meeting **June 10 - 12, 2002**. You will be notified if the NCAB does not concur with the recommendations of the IRG. The percentile ranking and/or priority score are not the only factors considered in NCI funding decisions. Therefore, you should make no assumptions about the probability of funding. **If you would like further information concerning your chances for funding, please call the program director listed on your Summary Statement.** Please note that further staff evaluation may result in the funding of grants at less than the IRG recommended levels or for shorter periods of time.

This percentile ranking and/or priority score are shown on the Summary Statement. Certain types of applications, such as program projects, RFA responses, and small business applications, are not percentiled and receive only a priority score. The priority score is determined by averaging the scores given individually and privately by each voting member of the Initial Review Group (IRG). Priority scores range from 100 to 500, with 100 representing the greatest scientific merit. The percentile ranking is the relative ranking of an application compared with all other applications reviewed by the same IRG. The lower the percentile ranking, the higher the scientific merit.

We hope that this information will be helpful to you in your present and future research activities. If the Summary Statement raises questions which you believe require our attention, it is important that you contact the Program Director listed on your Summary Statement as soon as possible, but no later than 30 calendar days following the NCAB meeting.

Sincerely,

*Tracy Lugo, Ph.D.*  
Program Director  
Cancer Diagnosis Program  
Phone: 301-496-1591  
Fax: 301-402-7819  
E-Mail: tl82s@nih.gov

Enclosure

cc: Business Official (letter only)

TRACY LUGO PH.D.  
301-496-1591  
tl82s nih.gov

SUMMARY STATEMENT  
( Privileged Communication )

Release Date: 02/20/2002

Application Number: 1 R21 CA097208-01

ACHARY, PATNALA M  
ALBERT EINSTEIN COLLEGE OF MED  
DEPT OF RADIATION ONCOLOGY  
1300 MORRIS PARK AVENUE  
BRON , NY 10461

Review Group: PTHB  
Pathology B Study Section

Meeting Date: 01/28/2002  
Council: MAY 2002  
Requested Start: 07/01/2002

R A PA: PA01-010  
PCC: 4GDR

Project Title: Markers of Metastasis in Ductal Mammary Carcinoma

SRG Action: Priority Score: 236 Percentile:  
Human Subjects: 30-Human subjects involved - Certified, no SRG concerns  
Animal Subjects: 30-Animals involved - no SRG comments or concerns noted  
Gender: 2A-Only women, scientifically acceptable  
Minority: 1A-Minorities and non-minorities, scientifically acceptable  
Children: 4A-Child representation unknown, scientifically acceptable  
Clinical Research - not NIH-defined Phase III Trial

Project Year	Direct Costs Requested	Estimated Total Cost
1	100,000	163,650
2	100,000	163,650
<b>TOTAL</b>	<b>200,000</b>	<b>327,300</b>

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

NOTE TO APPLICANT FOLLOWS SUMMARY STATEMENT

NEW INVESTIGATOR

**RESUME AND SUMMARY OF DISCUSSION:** Further consideration with the requested budget for two years is recommended for this R21 application. The strength of this application is the initial isolation of a sequence whose deletion seems to track with lymph node metastasis although the screen is very limited. The weaknesses are a poorly developed plan to determine clinical correlations, a poorly developed plan to identify the genes linked to the deletions and a premature proposal to investigate any putative gene in murine models of metastasis.

**DESCRIPTION (provided by applicant):** The objective of this proposal is to identify molecular genetic markers for detecting ductal mammary carcinomas that are prone to developing metastases. The hypothesis to be tested is that in order to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic changes in addition to those that led to transformation. This may involve the loss of function of metastasis suppressor genes or the activation of metastasis promoting genes such as oncogenes. The existence of both types of genes have been reported in several tumor types, but many more are very likely to exist, and much remains to be learned about the possible roles of such genes in mammary carcinoma.

A subtractive DNA hybridization technique, Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary tumor with that of the metastatic lymph node of the same patient to isolate those sequences that were lost and also gained in the course of tumor metastasis. The tumor and metastatic cells were recovered by microdissection methods to recover a large number of pure tumor cells required for RDA.

We have isolated about 11 candidate metastasis associated gene sequences (MAGS) that were found to be lost in metastatic cells. When screened on normal, primary and metastatic cell DNA samples from 5 breast carcinoma patients one of them (MAGS-XI) was found to be lost in the metastatic cells of 3 out of 5 patients and another sequence (MAGSIX) in 2 out of 3 patients indicating their involvement in breast metastasis.

The first specific aim is to confirm whether or not the remaining differential products from the RDA experiments performed previously are consistently lost in the index breast carcinoma cases used for each RDA. The second aim is to screen additional 30 patient samples using previously isolated MAGS and the candidate MAGS that will be identified in specific aim 1, to determine if these sequences are consistently associated with metastasis by PCR/Southern blotting methods. The third specific aim is to isolate partial or full-lengths of the MAGS and use as fluorescence in situ hybridization (FISH) probes to screen a large number of patient tissue samples, to reinforce the findings of specific aim 2 and to generate full-length genes for transfection experiments (Specific aim 4). The fourth aim is to transfect cells with selected MAGs and to evaluate their metastasis potential in vivo using our SCID mouse model of breast metastasis.

The long-term goal of the proposed study is to identify a panel of genes that could be used for better prognosis as well as the basis for the development of better therapies in future.

#### **CRITIQUE #1:**

**SIGNIFICANCE:** This is a proposal to identify genes that are mutated during breast cancer progression and correlate those mutations with lymph node metastasis. This goal is of major importance both for our understanding of the mechanisms of cancer progression and also potentially for developing clinical strategies for breast cancer.

**APPROACH:** This is a proposal for an R21 grant from Dr. Achary who intends to use RDA to isolate a series of genes that are deleted during breast cancer progression. The preliminary data indicates the potential feasibility of this approach. They have performed RDA using DNA from breast carcinoma

lymph node metastases compared to DNA from the normal tissue or primary tumor counterparts from the same patient. Only the RDA clones from the comparison between the metastatic lymph node and its normal counterpart are shown in the application. The most likely candidate as described in the application is MAGS-IX. Currently the presence of MAGS-IX was decreased in 3 out of 4 cases examined in the lymph node metastases compared to primary tumor tissue or normal breast epithelium. This sequence has resisted sequencing so far. (A minor problem with the application was the frequent designation of MAGS-IX as MAGS XI. The reviewers presume that this is a typo and that there are not 2 clones. If there are two clones the interchanging of the discussion of each would have been totally confusing.)

Based upon these preliminary results the applicants propose 4 specific aims. The first two will extend the preliminary observations by continuing to characterize the RDA clones already isolated. This aim overlaps with specific aim 2 that is to confirm the loss of the sequences in breast cancer lymph node metastases using PCR and/or DNA blotting. Obviously this must be done to determine whether these clones will truly lead to genes whose sequences are deleted during breast cancer progression.

The problem that many workers have encountered in attempting to use RDA to identify genomic regions frequently affected during cancer progression has been the variability between individuals so that tumors cannot be compared to DNA from another individual. The applicant has avoided that problem by using tumors and normal tissues from the same person and this is one of the strengths of the application. They do not discuss the problem that might arise if there was microsatellite instability in the tumor so that many changes might arise, but those changes might have no causal effect on the tumor phenotype.

A point not discussed was who was actually going to do the microdissection and confirm the histopathology of the cells dissected. There is a letter of collaboration with a pathologist, but this suggests that the pathologist will teach a technician to identify the cells. There would be a need for assurance that the cells taken for analysis were truly tumor cells.

To evaluate the significance of the candidate deletions it will be necessary to evaluate various breast cancer specimens for their potential loss of the putative suppressor sequences. A significant problem with the proposal is the very limited discussion of the necessary clinical parameters needed to develop any meaningful clinical conclusions. The authors need to be aware that lymph node metastasis and hematogenous metastasis have markedly different clinical implications. Further, considerations based upon the number of lymph nodes involved affect prognosis as well as different methods of identifying lymph nodes- for example, sentinel node involvement- must be taken into consideration. None of the clinical considerations that affect patient outcome or the different therapies that the various patients might encounter are discussed in the context of the experiments and the multivariate analysis that would be expected to be used. There is a letter of collaboration from a statistician, but no discussion of the approaches to be used.

Specific aim 3 is to identify the actual gene whose function is disrupted by the deletion and specific aim 4 to determine if the expression of these genes will disrupt metastasis in murine models of metastasis.

In specific aim 3 the applicants do not describe possible methods for determining the gene that is presumably affected by the deletions. This is not trivial since the gene may not be the coding region most proximal to the sequence identified by RDA.

Specific aim 4 is premature in the absence of any isolated gene and suffers from the problem that the genes isolated will have been identified because they may contribute to lymph node metastasis yet the model is for hematogenous metastasis.



**INNOVATION:** If successful this would open up new ways to identify important genetic changes in cancer and allow the identification of changes that specifically contribute to tumor progression.

**INVESTIGATOR:** Dr. Achary is an assistant professor in the department of radiation oncology at the Albert Einstein College of Medicine His training has been in cytogenetics and is now beginning to study various methods of gene profiling in cancer.

**ENVIRONMENT:** Suitable for the studies proposed.

**CRITIQUE #2:**

**SIGNIFICANCE:** This R21 application is in response to the program announcement of "Exploratory Studies in Cancer Detection, Prognosis, and Prediction". It is aimed at identifying molecular genetic markers for prediction of the metastatic potential of ductal mammary carcinomas. In this application, the P.I. proposed four specific aims to identify metastasis-associated markers. The proposed studies, if successful, may provide some novel markers as promising new molecular indicator of metastasis in breast cancer progression. It may provide some insight on the molecular mechanisms of genes involved in metastasis and may facilitate therapy and prognosis prediction of breast cancer patients.

**APPROACH:** This proposal stems from the P.I.'s interesting preliminary results. The P.I. has isolated several metastasis associated gene sequences (MAGS) in breast carcinoma by using RDA (Representational difference analysis). The P.I. proposes to screen a larger population of breast carcinoma patient samples to determine if these MAGS can be used as prognostic markers for the lymph node negative breast carcinoma patients who are prone to develop metastasis. In order to identify and confirm these new metastasis-associated markers, the P.I. proposed four Specific Aims. However, the reviewers have the following concerns on the proposed studies.

In the preliminary result section, the P.I. tried to screen patient samples with candidate MAGS-IX, IV, XI. However, only 4 cases were performed with MAGS-IX. MAGS IV and MAGS-XI detection in patient samples was not very successful (P 36). Based on these preliminary data, it is difficult to evaluate the potential value of these candidate metastasis-associated genes.

In the supplementary data, the P.I. demonstrated that additional breast tumor samples showed loss of MAGS-IV. However, they used the primary tumor and matched normal tissue DNA as a pair of comparison. Although they observed the loss of MAGS-IV in 2 tumor samples, this could not confirm MAGS-IV as a metastasis associated gene. Instead, MAGS-IV may simply be involved in tumorigenesis rather than metastasis.

In the RDA experiment design, the P. I. compared the primary breast carcinoma with lymph node metastasis for genetic difference. The P.I. was able to find gene loss in the metastasis and named them MAGS. However, as the P.I. mentioned in the proposal, the genetic alterations leading to metastasis might have already taken place in the primary tumor cells. Therefore, some of the genetic changes in metastasis may not be causative of metastasis but may only be the consequence of metastasis. A better way for identifying genes predicting metastasis of node negative patients is to compare tumor DNA from patients who are node-negative at diagnosis, but develop metastasis during follow-up with patients who are node negative and have no metastasis after long-time follow-up. The genetic difference between these two groups should be useful for identify node negative patients who may later develop metastasis.

As mentioned by the P.I. in the proposal, the goal of this application is to identify a panel of molecular genetic markers as prognostic markers for the lymph node negative breast carcinomas that are prone



to develop metastasis. These node-negative patients do not have metastasis at diagnosis, but later develop local or distant metastasis. It is important to identify these patients before metastasis happens so that they can receive more appropriate therapy. However, most of the markers in the proposal were lost in the metastatic lymph node, but retained in primary tumors (See Fig 9 and P36, "MAGS IX missing in lymph node metastatic cell foci but present in primary tumor cells"). This makes it difficult to use these genes for predicting early metastasis. It will be more valuable if the P.I. can find genetic changes that are different in the primary tumor among patients who had metastasis and who did not have metastasis.

The P.I. mentioned but not emphasized about the follow-up information in the proposal. It will be very important to observe if there exists any difference in metastasis incidence between patients with and without expression of MAGS in their primary tumor.

This is an over ambitious proposal for an R21 application. The P.I. proposed four specific aims in the proposal. Some experiments are quite time-consuming. For example, to collect 10,000 metastatic cells by single-cell microdissection (SCM) using mechanical microdissector takes much time. The P.I. needs to prioritize his research plan.

**INNOVATION:** In this proposal, the P.I. combines several powerful techniques to isolate molecular markers for breast cancer metastasis. These markers might be used for prognosis of node negative breast cancer patients. Furthermore, knowledge of genes involved and their function may identify novel targets for pharmacologic, genetic, or other therapeutic interventions for breast cancer.

**INVESTIGATOR:** Dr. Achary, his research team and collaborators are highly experienced in the proposed studies. They should be able to carry the proposed investigation successfully.

**ENVIRONMENT:** The available facilities at the Albert Einstein College of Medicine are excellent for conducting the proposed research.

**OVERALL EVALUATION:** This R21 proposal describes an important research to identify molecular markers for identifying node negative breast cancer patients who will develop metastasis. The preliminary results on some putative novel metastasis associated genes are very interesting. The experimental plan was over ambitious for an R21 application. There are some additional concerns on the research design as discussed above.

**GENDER, MINORITY AND CHILDREN SUBJECT:** Appropriate for the studies proposed.

**HUMAN SUBJECTS:** There were no concerns.

**ANIMAL WELFARE:** The P. I. has CCI (Committee on Clinical Investigations of Albert Einstein) approved animal use protocol.

**THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW ADMINISTRATOR TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:**

**PROTECTION OF HUMAN SUBJECTS (Resume): ACCEPTABLE.** A total of fresh frozen ductal breast cancer tissue from 120 cases will be analyzed in this investigation. These samples consist of primary tumor, positive lymph nodes and normal tissue and/blood samples from individual patients. Computerized case histories of all the cases including data on the patients' pathological parameters, radiosensitivity of the tumor, response of tumor metastasis to chemotherapeutic drugs and family

history with respect to ovarian and other cancers, etc. will be available to this investigation. However, this information is made available to the investigators in such a manner that subjects of this study cannot be identified directly or through linking identifiers.

**INCLUSION OF MINORITIES PLAN (Resume): ACCEPTABLE.** Samples are selected without any bias for race or age. Most importantly, the PI and the laboratory staff will be blinded with respect to the clinical response of the patients during the molecular studies. Successful completion of the proposed studies will lead to prospective studies in future grant projects that will assess genetic and environmental factors that relate to race, including attempts to understand why breast cancer has higher frequency and severity in women or certain groups.

**INCLUSION OF WOMEN PLAN (Resume): ACCEPTABLE.** Only women will be studied. Samples will be selected without any bias for ethnicity.

**INCLUSION OF CHILDREN PLAN (Resume): ACCEPTABLE.** Breast cancer is more frequently found in women over the age of 18, children will not be included in this study.

**VERTEBRATE ANIMAL (Resume): ACCEPTABLE.** Human mammary adenocarcinoma cell lines used to generate primary tumors and metastases in female immunocompromised mice (Rag-2 <sup>-/-</sup> or scid/scid). They estimate that up to 100 mice per year might be used. For each cell line to be tested, up to 10 mice will be used to generate lung metastases by injection i.v., and 10 mice being used to generate primary tumors in the mammary fat pad and metastases that develop from the primary fat pad. The human cell lines used will be MB-MDA-231 and MB-MDA-435. These cell lines are widely utilized and have shown no effects or risk to personnel. They estimate that up to 2 metastasis associated genes will be tested per year by transfecting these cell lines ((2 genes vector control) x 2 cells lines x 2 models x 10 mice per group) 100 mice per year. For the intravenous injection model, mice will be anesthetized with isoflurane delivered through a precision vaporizer. 100,000 - 500,000 cells suspended in 0.2 ml of media will be injected via the tail vein through a 26 guage needle. Mice will be monitored for signs of dyspna, weight loss, and failure to groom and will be sacrificed after 4 weeks or earlier if they show signs of respiratory distress or weight loss exceeding 20% of starting weight. For injection into the mammary fat pad, mice will be gently restrained and then 0.1 - 0.3 ml of MEM containing 500,000 cells will be injected in the mammary fat pad. The animals will then be housed in the animal facility for 6-10 weeks for generation of primary tumors and metastases. For generation of lung metastases, the primary tumor will be removed after the tumor reaches 2 cm in diameter. In this case, the animal will be anesthetized with isoflurane, the primary tumor removed surgically, and then the wound closed with sterile wound clips. The animals will then be allowed to continue for an addition 4 weeks to allow lung metastases to grow, or until the animal displace clear signs of metastases, such as dyspnea at which time the animal will be sacrificed by carbon dioxide overdose and tissues collected for analysis. To monitor the stress on the animal during tumor or metastasis growth, the animals will be checked by the Research Assistant from their lab every day. Four full-time veterinarians participate in their institutions' program of care and use. Veterinary care includes a program for prevention of disease, daily observation and surveillance for assessment of animal health; appropriate methods of disease control, diagnosis, and treatment; guidance of animal users in appropriate methods of handling, restraint, anesthesia, analgesia, and euthanasia; and monitoring of surgical programs and post-surgical care.

**COMMITTEE BUDGET RECOMMENDATIONS:** The budget was recommended as requested.

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NOTICE: The NIH has modified its policy regarding the receipt of amended applications. Detailed information can be found by accessing the following URL address:  
<http://grants.nih.gov/grants/policy/amendedapps.htm>

NIH announced implementation of Modular Research Grants in the December 18, 1998 issue of the NIH Guide to Grants and Contracts. The main feature of this concept is that grant applications (R01, R03, R21, R15) will request direct costs in \$25,000 modules, without budget detail for individual categories. Further information can be obtained from the Modular Grants Web site at <http://grants.nih.gov/grants/funding/modular/modular.htm>

## MEETING ROSTER

### Pathology B Study Section Oncological Sciences Integrated Review Group CENTER FOR SCIENTIFIC REVIEW

PTHB 1

January 28, 2002 - January 30, 2002

#### CHAIRPERSON

FEARON, ERIC R., MD, PHD  
ASSOCIATE PROFESSOR  
DEPARTMENT OF INTERNAL MEDICINE  
SCHOOL OF MEDICINE  
UNIVERSITY OF MICHIGAN  
ANN ARBOR, MI 481090638

HERLYN, MEENHARD F., DVM •  
PROFESSOR AND CHAIRMAN  
PROGRAM OF MOLECULAR/CELLULAR BIOLOGY  
WISTAR INSTITUTE  
PHILADELPHIA, PA 19104

#### MEMBERS

BAND, VIMLA, PHD  
PROFESSOR AND DIRECTOR  
RADIATION & CANCER BIOLOGY DIVISION  
DEPARTMENT OF RADIATION ONCOLOGY  
NEW ENGLAND MEDICAL CENTER  
TUFTS UNIVERSITY SCHOOL OF MEDICINE  
BOSTON, MA 02111

ISOM, HARRIET C, PHD •  
ASSISTANT DEAN AND DIRECTOR OF THE MD/PHD  
PROGRAM  
DISTINGUISHED PROFESSOR OF MICROBIOLOGY AND  
IMMUN  
AND PROFESSOR OF PATHOLOGY  
DEPT. OF MICROBIOLOGY AND IMMUN. ROOM C6716  
PENN STATE COLLEGE OF MEDICINE  
500 UNIVERSITY DRIVE  
HERSHEY, PA 170330850

BECKER, DOROTHEA, PHD •  
PROFESSOR  
DEPARTMENT OF PATHOLOGY  
UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE  
RM BST 1-E 1050  
211 LOTHROP STREET  
PITTSBURGH, PA 15213

ITZKOWITZ, STEVEN H., MD •  
THE DR. BURRILL B. CROHN PROFESSOR OF MEDICINE  
DIRECTOR, THE DR. HENRY D. JANOWITZ DIV. OF  
GASTRO  
ACTING CHAIR, THE SAMUEL BRONFMAN DEPT OF MED  
MOUNT SINAI SCHOOL OF MEDICINE  
BOX 1069  
ONE GUSTAVE LEVY PLACE  
NEW YORK, NY 100296574

CHANG, DAVID D., MD, PHD  
ASSOCIATE PROFESSOR  
DEPARTMENT OF MEDICINE  
DIVISION OF HEMATOLOGY/ONCOLOGY  
UNIVERSITY OF CALIFORNIA, LOS ANGELES  
LOS ANGELES, CA 90095

KERN, SCOTT E., MD  
ASSOCIATE PROFESSOR  
DEPARTMENT OF PATHOLOGY  
JOHNS HOPKINS UNIVERSITY  
SCHOOL OF MEDICINE  
BALTIMORE, MD 212052195

CHIN, LYNDIA, MD •  
ASSOCIATE PROFESSOR OF DERMATOLOGY  
DANA-FARBER CANCER INSTITUTE  
HARVARD MEDICAL SCHOOL  
44 BINNEY STREET  
M413  
BOSTON, MA 02115

LANGUINO, LUCIA R., PHD  
ASSOCIATE PROFESSOR  
DEPARTMENT OF PATHOLOGY  
SCHOOL OF MEDICINE  
YALE UNIVERSITY  
NEW HAVEN, CT 06510

DE CLERCK, YVES A, MD •  
PROFESSOR  
DEPARTMENT OF PEDIATRICS  
CHILDREN'S HOSPITAL OF LOS ANGELES  
DIVISION OF HEMATOLOGY/ONCOLOGY  
LOS ANGELES, CA 90027

LLOYD, RICARDO V, PHD, MD •  
PROFESSOR OF PATHOLOGY  
LABORATORY OF MEDICINE AND PATHOLOGY  
DIVISION OF ANATOMIC PATHOLOGY  
MAYO CLINIC AND FOUNDATION  
ROCHESTER, MN 55905

HENDRIX, MARY J., PHD •  
KATE DAUM RESEARCH PROFESSOR AND HEAD  
DEPARTMENT OF ANATOMY AND CELL BIOLOGY  
DEPUTY DIRECTOR AND ASSOC. DIRECTOR OF BASIC  
RES.  
HOLDEN COMPREHENSIVE CANCER CENTER  
THE UNIVERSITY OF IOWA  
IOWA CITY, IA 52242

MC CARTHY, JAMES B., PHD •  
PROFESSOR  
DEPARTMENT OF LABORATORY MEDICINE  
AND PATHOLOGY  
UNIVERSITY OF MINNESOTA CANCER CENTER  
MINNEAPOLIS, MN 55455

**Introduction (revised application)**

In this revised application the objective and long-range goal remain the same as presented in the original submission. However, we have made significant changes in the specific aims and research design based on the comments and recommendations of the reviewers. We omitted certain time consuming methods and replaced them with simple methods and strategies to achieve expected objectives within the scheduled time frame of this R-21 grant.

The main changes are as following:

1. Specific aims are reduced from 4 to 2. The main aim of the project is presented in the specific aim 1 {To isolate a panel of DNA sequences for distinguishing primary tumors that did develop metastasis (Group-I) from those (Group-II) did not develop metastasis} by testing a series of 40 patient samples from each group, whose clinical outcome is known. The specific aim 2 is to test a limited number of BACs containing metastasis associated DNA sequences (MADS) to determine if they have inhibitory effect on metastatic potential using functional studies. Attempts to isolate metastasis associated gene(s) that may be present in the deleted region indicated by each MADS have been omitted in this application.
2. Based on the clinical parameters, screening of 15 MADS using Q-Rt-PCR and FISH methods on 40 primary tumors which developed lymph node metastasis and another 40 which did not develop metastasis are proposed.
3. For FISH screening of tumor samples, complete BACs containing MADS will be used instead of long DNA fragments or full-length cDNA sequences as FISH probes.
4. Q-Rt-PCR screening of the sample samples is proposed to strengthen the FISH results and vice versa.
5. For functional studies only spontaneous metastasis (Mammary fat pad) model is proposed and hematogenous model was omitted. We will examine the regional lymph nodes and lungs for gross and histologic evidences of metastasis.

Note: Please note that what we previously designated as metastasis associated gene sequences (MADS) are referred to as metastasis associated DNA sequences (MADS)

**Response to Critiques****Critique 1**

**Page# 2: Approach: 1<sup>st</sup> paragraph** (This is a proposal for an R-21 grant from.....): Please note that MADS-IX and -XI are different. The MADS-IX could be sequenced and the sequence derived primers yielded a PCR product of the correct size without any non-specific bands. On the other hand MADS-XI has several dinucleotide repeats and was difficult to sequence at first. We therefore used the whole sequence of MADS-XI as p32-dCTP probe to screen a slot blot containing DNA from patient samples. However, we later obtained the complete sequence of the MADS-XI (224bp) and presented additional data about this sequence in the preliminary findings section of this revised application.

**Page# 3: Approach: 2<sup>nd</sup> paragraph** (Based upon these.....): We agree with the comments of the reviewer. Instead of 4 specific aims, we now have 2 specific aims.

**Page# 3: Approach: 3<sup>rd</sup> paragraph** (The problem that.....): As pointed out by the reviewer there will be MSI in the tumors and we are aware of the genetic variation this may cause. However MSI in breast cancer is not as common as colon or pancreas cancers (Lee et al. 2001) and MSI alterations in the coding regions of breast cancer are rare compared to colorectal cancers (Forgacs et al. 2001). While MSI has been shown in a small fraction of sporadic breast tumors (0-30%), its frequency varies from 0% (Lothe et al. 1993) to 83% (Glebov et al. 1994) in familial cases. Since our studies are restricted to sporadic breast cancer patient samples only, the MSI variations may not be significant in our studies. Furthermore, to reduce chances of pursuing those differences resulting from MSI, we made a criteria that for a RDA sequence to be considered as a candidate MADS, it has to be present in normal cell DNA, missing in primary tumor cell DNA (heterozygous loss) and in tumor cells of the lymph nodes (heterozygous/homozygous loss) in at least 2 cases (It is unlikely that the MSI will be in exact same place in 2 unrelated cases).

**Page# 3: Approach: 4<sup>th</sup> paragraph** (A point not discussed who.....): Microdissection is one of the very important components of this application. Cell purity must be about 95% for RDA and we had no access to LCM in the beginning of the project so we used single cell microdissection (SCM) which can provide 99% purity of tumor cell population. However later we switched to laser capture microdissection (LCM) for all the RDA and Q-Rt-PCR analyses. For the LCM, Dr. Jones, a board certified pathologist and Dr.Klinger who did a residency in pathology, first identify and mark the



tumor cell islands on the slides. The PI and the Research Associate, who by now also have considerable experience recognizing tumor cells, then use LCM to pick up the cells from the marked areas. Please see Dr. Jones's letter of collaboration.

**Page# 3: Approach: 5<sup>th</sup> paragraph** (To evaluate the significance.....): As outlined in the application we have now collected 120 ductal mammary carcinoma samples for which we have at least 5 year morbidity, mortality, pathohistologic information, age, course of treatment, lymph node status, ER/PR and Her2/neu status. We now know that about 50 of these cases of primary carcinomas developed metastases and the remaining 70 did not develop metastases within 5 years of primary tumor detection. Additional samples (about 100 tumor samples with corresponding negative or positive lymph nodes) with similar clinical information are being provided by Dr. Wahab of Cairo University (See Dr. Wahab's letter). Our pathologist has clarified to us the different prognostic indicators of lymph node versus hematogenous metastasis and she also explained the different methodologies for assaying lymph node status. In this proposal we decided to omit metastasis cases assessed by sentinel lymph node sampling. The P.I., the Pathologist and the Biostatistician have extensively discussed on the appropriate clinical parameters and suitable statistical analyses to be performed in this study (See details in the methods section; Pages. 36-37).

**Page# 3: Approach: 7<sup>th</sup> paragraph** (In specific aim 3 the applicants.....): RDA yields sequences which could be partial sequences of metastasis associated genes, or sequences of genes of unrelated function as well as non coding regions on the same deleted segment. It is very important to point out that in either case they could serve as markers if they are consistently missing in a high proportion of cases and are associated with known clinical outcome. We found that out of 11 MADS, 2 were expressed sequences and 9 were intronic sequences. Both types may be parts of either metastasis associated genes, or sequences of unrelated function. We noted in the original application that to obtain full-length of genes (with exons and/or introns) inverse PCR method will be used, if the MADS are intronic sequences. Similarly full length cDNAs will be obtained by screening cDNA libraries if the MADS are exonic sequences. In this revised submission we are not proposing any attempts to obtain full-length of genes. We are also not planning to analyze the deleted region with STS markers to map the region and find out the nearby genes that might play a role in metastasis. Instead, we are focusing on the screening of these 15 MADS (previous 11 and new 4) on 80 patient samples (Group-I=40; and Group-II=40) with BACs containing MADS. One or two MADS which are most promising in that they are missing in the highest number of patients in group- I, will be selected and the retrofitted BACs containing those MADS will be used directly for functional studies. If metastasis inhibitory effects are observed at in vitro or in vitro studies, then a separate grant will be applied to dissect the whole insert of the BAC clone to identify the actual metastasis suppressor gene or genes in that region. Dr. Athwal of Temple University has agreed to collaborate on this aspect once the MADS are selected (See Dr. Athwal's letter).

**Page# 3: Approach: 8<sup>th</sup> paragraph** (Specific aim 4 is.....): We agree that the specific aim 4 is premature in the absence of any isolated genes. We proposed this aim because we already had isolated couple of positive clones for MADS-IV and -X by screening a cDNA breast library and a partial gene sequence with intronic sequence (MADS-IX) using inverse PCR methods. To determine the function of these clones we wished to evaluate the metastatic inhibition in the mouse model. Therefore we established the in vivo mouse metastasis model and successfully tested by using metastatic MDA-MB-435 mammary carcinoma cell lines transfected with nm23 (metastasis suppressor) and in another experiment transfected with ErbB2 (metastasis enhancer). In our original application we proposed 2 mouse models (spontaneous and experimental metastasis) for the evaluation of the metastatic potential of the gene sequences that are expected to be isolated in this investigation. While the first model (2 left side panels in the figure) is the mammary fat pad model which could be used for evaluation of both tumorigenesis and metastasis, the second model (third panel) could only be used for hematogenous metastasis. As pointed out by the reviewer the second model is not appropriate for this investigation so we will not use it. We will use the spontaneous metastasis model and examine the lymph nodes for metastasis in the mice as well as examining the metastasis in lungs (See Dr. Russel's letter).

## Critique #2

**Page# 4: Approach: 2<sup>nd</sup>** (In the preliminary result section.....) **and 3<sup>rd</sup> paragraphs** (In the supplementary data.....): In the original application we indicated that MADS-IV was missing in 1 out of 3 tumor cell lines. Since we did not have a counterpart metastatic cell DNA we could not determine if the sequence is also missing in metastatic cell DNA. However we predicted that it must be missing in metastatic cell DNA because it was lost homozygously in the tumor DNA. However as shown in the supplementary data section, PCR screening of 9 patient tumors showed that it is lost

heterozygously in one case and homozygously lost in another case indicating that this MADS maybe associated with either tumorigenesis or metastasis or both. The results for MADS-XI was particularly interesting. In 1 out of 5 cases the primary tumor shows heterozygous loss and metastatic cell DNA shows homozygous loss. In 3 out of the 4 remaining cases there was homozygous loss of MADS-XI in the metastatic DNA. In the remaining one case there was no loss of MADS-XI, either in the primary or metastatic DNA.

**Page# 4: Approach: 4<sup>th</sup> paragraph** (In the RDA experiment design.....): We liked the suggestion of the reviewer that a better way for identifying genes predicting metastasis of node negative patients is to compare tumor DNA from patients who are node-negative at diagnosis, but develop metastasis during follow-up with tumor DNA of patients who are node negative and have no metastasis after long-term follow-up. The advantages are, that one can identify losses occurring in primary tumor that lead to metastasis. It reduces the likelihood of detecting losses occurring after metastasis that are unrelated to the metastasis process. However, to undertake such a comparison we have the following concerns: Firstly, in RDA one cannot directly compare tumor DNA from one patient with tumor DNA from a different patient due to obvious reasons (polymorphic variations etc.). Secondly, a large number of products associated with tumorigenesis and genome instability may be isolated. Thirdly, chances of failing to detect MADS because of low percentage of primary cells with the loss among the microdissected cell population or chances that the biopsied tumor cells are from early stage in which such changes leading to metastasis have not yet occurred. Anticipating such a situation in our original proposal we compared normal cell DNA or primary cell DNA with metastatic cell DNA expecting to find genetic differences that took place in the transition from normal to primary to metastasis. Then identify the genetic differences between primary and metastasis by screening normal, primary and metastatic cell DNA samples of additional patients. Once confirmed that they are associated with metastasis, they could be used to screen biopsy samples of primaries that subsequently developed metastasis and those primaries that did not develop metastasis to see if they could be differentiated with the help of these panel of markers.

**Page# 4: Approach: 5<sup>th</sup> paragraph** (As mentioned by the P.I. in the proposal.....): As pointed out by the reviewer, if a sequence is lost in metastatic cells but retained in the primary tumor it will make it difficult to use the marker for predicting metastasis. However, our preliminary results suggest a different situation. For example, MADS-IX is homozygously lost in 2 out of 3 metastatic cell DNA samples, suggesting its association with metastasis. With regard to the corresponding primary tumor DNA samples, this sequence is heterozygously lost in those 2 cases. We believe that this result is useful because by finding a loss of heterozygosity of this sequence in primaries (which lead to complete loss in metastasis) we could use it as a marker for metastasis. If this situation is consistently observed for a MADS in the group-I, then it could be used as a predictive marker for metastasis.

**Page# 5: Approach: 6<sup>th</sup> paragraph** (The P.I. mentioned.....): To study the expression of MADS, firstly the MADS should be an expressed sequence preferably a part of the functional gene. Secondly, the primary tumor cells have to yield RNA for performing RT-PCR or Northern blotting. In our situation, 2 out of 11 MADS were found to be expressed which could be used for RT-PCR. However since the primary tumors are paraffin embedded archival tissue samples it is very difficult to obtain total RNA/mRNA for expression studies. However eventually, if antibodies are available we can look for the corresponding protein expression in archival tissues.

**Page# 5: Approach: 7<sup>th</sup> paragraph** (This is an over ambitious.....): We reduced 4 aims to 2 aims and focused on the screening of about 80 patient samples (Group-I and II) with the existing MADS to determine the strength of association. Attempts to isolate full-length of genes for functional studies are not proposed. Instead, to determine if these metastasis suppressor genes are present in the deletion encompassing the MADS, we proposed to use a short-cut method to evaluate the inhibitory potential of metastasis in only 2 or 4 of the promising MADS. This will be done by directly transfecting the retrofitted BACs containing the MADS into a highly metastatic cell line followed by In Vitro and In Vivo studies. Only mammary fat pad model (spontaneous metastasis) will be used instead of 2 models originally described. As pointed out by the reviewer, it is true that it will consume lot of time if 10,000 cells are microdissected by SCM. However, we mentioned in the application that we used SCM in the beginning when we had no access to LCM. Since last year we have our LCM facility and as you are aware that it takes only 30 minutes to dissect 10,000 cells by LCM. Now we have prioritized our experiments in the research plan to achieve the main aims of the project.



## Research Plan

### a. Specific aims:

**The long-term objective** is to develop a panel of DNA markers that could be used reliably to identify ductal mammary carcinomas that are prone to developing metastases (Group-I) from those that are not likely to metastasize (Group-II). Consequently attempts will be made in future studies to isolate metastasis suppressor genes, that are existing in the deletion region from which the marker sequence(s) are generated by Representational Difference Analysis (RDA). Isolation of novel genes associated with metastasis may identify novel targets for pharmacologic, genetic, or other therapeutic strategies and also should help elucidate the molecular mechanisms and pathways of genes involved in breast metastasis.

**Specific Aim 1:** To isolate a panel of DNA sequences for detecting primary tumors that did develop metastasis (Group-I) and those did not develop metastasis (Group-II). Previously we have isolated 11 MADS and three of these were shown to be missing in samples of metastasis from additional patients. From subsequent 10 RDA experiments using the 'total probe' method we have identified 4 additional groups of MADS. We propose to screen candidate MADSs step-wise by quantitative real-time PCR (Q-Rt-PCR) and by fluorescence in situ hybridization (FISH) methods on primary tumors of 40 patients who developed metastases in the lymph nodes (Group-I) and 40 patients who did not develop metastases in the lymph nodes within 5 years of primary tumor detection (Group-II). Tumor cells will be isolated by Laser Capture Microdissection (LCM) for Q-Rt-PCR analysis. We have designed primers for all the MADSs for Q-Rt-PCR and also identified BACs (except MADS-IV which has no homology in gene banks) that contain respective MADSs and also currently using the whole BACs as FISH probes.

**Specific Aim 2:** To determine if the BACs containing the MADS have inhibitory effect of metastasis using functional studies. Highly metastatic MDA-MB-435 human mammary carcinoma cells will be transfected with the retrofitted BAC containing MADS of interest. *In vitro* and also *in vivo* mammary SCID mouse model will be used to determine if the highly metastatic cell line transfected with the BAC/MADS changes to low or non-metastatic phenotype.

**Hypothesis:** In order to achieve the metastatic state, a primary mammary carcinoma cell must acquire genetic changes in addition to those that led to transformation. This may involve the loss of function of genes that inhibit the cell from being invasive, or the activation of metastasis promoting genes. The loss of function of genes is due to mutations and/or partial or complete loss of the genes due to deletions resulting from breakage or unequal crossing over etc. These deleted large DNA segments may harbor genes that may inhibit metastasis as well as DNA sequences with unrelated functions. Since RDA products are a part of the deletion, it may provide DNA sequences from unrelated genes or partial sequences of the metastasis suppressor genes. Any of these could be used as markers in the prognosis of the breast metastatic disease and could help to identify segments of the genome containing functional genes related to the metastatic potential.

### b. Background and significance:

The clinical outcome is generally positive for patients with node-negative breast carcinoma (i.e., those who do not have detectable metastases in the lymph nodes) who have been treated with surgery or surgery and radiation therapy. In about 13% of the patients, however, the disease spreads, and they are at risk of death (Lewis and Conry, 1992; Scorilas et al., 1993; Fisher et al. 1997). Genetic tests capable of identifying patients at risk for metastatic spread and/or better treatment targeted to eradicate metastatic tumor deposits could have a dramatic impact on the overall survival of these patients.

Two general types of genetic mechanisms are involved in the progression of a tumor to the metastatic state. One involves the loss of function of genes that prevent cells from becoming invasive. These are analogous to tumor suppressor genes, in which the loss of wild-type growth regulatory function leads to unregulated or malignant growth. Mutation or complete loss of the wild-type counterpart of any such gene in a malignant cell may cause the cell to become more motile, invasive and metastatic. For example, mutations of genes regulating cell adhesion molecules have been reported to be metastasis-fostering alterations (Pignatelli and Vessey, 1994; Shi et al., 1994; Tozawa et al., 1995; Li et al., 1997). A few such genes have been reported in different neoplasias namely Mdm2 gene in mouse uterine adenocarcinomas and PTEN gene in human brain, breast and prostate cancers (Risinger et al., 1994; Li et al., 1997). In human breast and mouse mammary tumors, several other genes have been shown to be associated with metastasis, including nm23 (Steeg et al., 1993), KAI1 (Dong et al. 1995; Guo et al. 1996; Yang et al. 2001), BRMS1 (Seraj et al. 2000) and mta1 (Toh et al., 1995). Just as there are many genes regulating cell mobility, adhesion, etc., there are probably many additional metastasis suppressor genes that

remain to be identified and characterized functionally. It is also likely that either loss or alteration of the function of several genes (at least 2) are necessary for a malignant cell to become invasive and then metastatic. The second category of genetic alteration involves a temporally inappropriate activation or overexpression of genes that promote metastasis.

This proposal is primarily targeted at the isolation of DNA marker sequences or sequences of genes that belong to the first category. The first aim of this application is to identify marker DNA sequences that are involved in the progression of metastasis so that a panel of molecular genetic markers for detecting those 13% mammary carcinomas that are prone to developing metastases could be identified earlier and treated more aggressively. There will also be considerable benefit to the women with mammary carcinomas that are not likely to metastasize (an estimated 87%; Lewis and Conry, 1992; Scorilas et al., 1993; Fisher et al. 1997). These women could then be treated more conservatively, sparing them the considerable physical, mental and financial costs of the treatment and with greater ease of mind than is possible today. The second aim will attempt to determine if these MADS are proximal to sequences which may have functional effects on metastatic phenotype. We will transfect the BACs containing the promising MADS to determine if the BAC insert sequence harbors metastasis supresor gene(s). However isolation and functional characterization of the involved gene(s) will not be possible in this project and requires a separate grant application.

Previous reports document a significant relationship between LOH and tumor suppressor genes (Cavenee et al. 1983; Cropp et al. 1990; Devilee et al. 1989; Sato et al. 1990) and metastasis associated genes (Hennessy et al. 1991; Lindblom et al. 1993) in breast cancers. A statistically significant increase in LOH events involving unknown genes on chromosome 16q (Lindblom et al. 1993) and on 14q (O'Connell et al. 1999) and metastasis in breast cancers are reported. Measuring LOH, O'Connell et al (1999) found that the majority of lymph node-negative primary breast tumors did not amplify a region linked to D14S62 and D14S51, while lymph node-positive breast tumors retained heterozygosity for these markers. These data could imply the existence of metastasis promoting gene(s) in that region or alternatively, the observed molecular changes may be used as a marker of metastatic propensity (Welch and Rinker-Schaeffer, 1999).

Breast metastasis disease is a complex process involving multiple steps and therefore global genetic profiling studies of metastatic and non metastatic tumors are required to elucidate this process. Currently we have access at Albert Einstein College of Medicine to the powerful tools like cDNA microarrays and proteomics to portrait the gene expression and protein profiling of tumors. However in the present investigation we are using archival material (mostly paraffin embedded tissue samples) which are not amenable to these methods. Therefore we proposed to use a robust genomic subtractive hybridization technique namely, RDA for isolating markers to differentiate the group-I primary tumors from group-II tumors. RDA is especially useful for identifying the loss (deletions) or acquisition of foreign DNA sequences (Risinger et al., 1994; Lisitsyn et al. 1995; Schutte et al 1995; Li et al., 1997). Since we are interested in DNA marker sequences or genes, whose loss is responsible for metastasis, we only focused on the loss products of RDA. This method is highly effective and does not require prior knowledge about the marker DNA sequences or the gene sequences one is searching for. The loss of function of genes is generally due to mutations and/or partial or complete loss of the genes in the form of deletions resulted from breakage or unequal crossing over etc. Since RDA products are a part of the deletion, it may provide DNA sequences from non-coding regions, unrelated genes or partial sequences of the metastasis suppressor genes. Any of these could be used as markers in the prognosis of the breast metastatic disease and could help to identify segments of the genome containing functional genes related to the metastatic potential. Once identified, markers can be used to test primary breast tumor biopsies, to identify the absence/presence of a sub group of cells in the primary tumors that are likely to metastasize.

The innovation in this proposal is the combination of 4 powerful technical resources to isolate molecular genetic markers specific to breast metastasis. Using archival tissue samples we can retrospectively correlate the isolated molecular markers with the clinical outcome of patients. Pure populations of tumor cells without contamination of stromal cells could be isolated by laser capture microdissection. Representational difference analysis (RDA) can isolate metastasis-specific genetic markers by comparing normal, primary and metastatic cell populations. The isolation of pure population of primary and metastatic cells from tissue samples is the main hindrance in undertaking such experiments. This has been circumvented by employing laser capture microdissection, a very fast method to procure cell populations with high purity. We proposed to use fluorescence in situ hybridization (FISH) method to screen primary tumors which could simultaneously detect the presence of MADS in normal and presence or absence in the neighboring tumor cells in the same tumor tissue section. We believe that this innovative research strategy, will provide the highest probability of success in identifying novel molecular markers specific to breast cancer metastasis. We have performed RDA experiments using 9

DMC cases and 2 cell lines and isolated 15 MADS. So far, on screening additional 11 patient samples we found that 3 of the MADS were missing in the DNA samples of the metastatic cell DNA of some of the patients. Thus our preliminary results are encouraging.

In this proposal the main focus is on the isolation of markers for breast metastasis. However if the BACs containing these MADS yield genes without any known function then a number of strategies could be used in future investigations to establish their function, similar to what was done with some of the genes so far known to be related to breast cancer development such as nm23, mta1, BRCA1, BRCA2, KAI1 and PTEN/MMAC1; BRMS1 (Steeg et al., 1993; Toh et al., 1995; Dong et al., 1995; Fitzgerald et al., 1996; Langston et al., 1996; Tavtigian et al., 1996; Li et al., 1997; Steck et al., 1997; Seraj et al. 2000; Yang et al. 2001). Even without cloning of the proposed metastasis related gene(s), these studies have the potential to provide a genetic test that could aid in diagnosing metastasis. However, knowledge of the sequence and normal function of putative metastasis suppressor gene(s) that we may find which could play an important role in metastasis, could ultimately lead to the development of pharmaceutical agents or gene therapeutic or other procedures for counteracting the adverse metastasis-promoting function of these genes in breast cancer.

### **c. Preliminary studies:**

In order to isolate metastasis associated DNA sequences (MADS) we have used genomic representational difference analysis (RDA), a subtractive hybridization method (Lisitsyn et al., 1995; Li et al., 1997), on human breast carcinoma tissue samples and cell lines. We used genomic DNA because we have been interested in identifying additional novel markers (gains or losses in metastasis) in archival tissues of patients whose outcome is known. In the first RDA experiment we therefore compared normal versus matched metastatic cell DNA of a ductal mammary carcinoma patient. Primary tumor sample from this case was too small to be used in a RDA experiment. In the remaining 8 genomic RDA experiments, however, we compared DNA samples from primary tumor cells with matched DNA of metastatic cells and successfully isolated differential sequences. Similarly RDA was performed in 2 pairs of mammary carcinoma cell lines (MDA-MB-435) with different metastatic potentials. Using 'total probe' method 15 groups of MADS have been isolated. Screening of 3 MADS in additional patient samples are promising and further characterization is in progress.

#### **1. Material used:**

In the preliminary studies, 20 archival ductal mammary carcinoma (DMC) tumor samples and 2 cell lines were used. They were collected from the Co-operative Human Tissue Network (C-1050; C-18805; C-98-05H; C-19898; C-20635), Cairo University (CU: 1-10) and Pathology Department of AECOM/MMC (DS-96-05); MDA-MB-435 cells (Low and high metastatic phenotypes by nm23 transfection) are gift from Dr. Patricia Steeg of NCI/NIH; MDA-MB-435 cells derived from primary and metastatic tumors in nude mouse (Price, 1996) are gift from Dr. J. Price of MD Anderson Medical Center, Houston; Paired cell lines derived from normal tissue/ blood and primary tumors of 3 breast carcinoma patients with known losses in specific chromosomal regions (HCC-1806; HCC-1143; HCC-1428; Gazdar et al. 1998) were received as gift from Dr. Ramon Parson of Columbia University, NY. The tumor cell line of the 4<sup>th</sup> pair of cell lines, has homozygous loss of PTEN gene (Li et al. 1997) and was obtained from ATCC (HCC-1937; Tomlinson et al. 1998).

#### **2. RDA experiments:**

We have performed RDA using DNA from archival tissues of normal tissue and matched metastatic lymph nodes of one ductal mammary carcinoma (DMC) case, and DNA from archival primary tumor and matched metastatic lymph nodes of 8 additional DMC cases. These 9 cases consisted of 3 cases each from poorly, medium and well differentiated tumor groups. Genomic RDA was also performed using low and high metastatic MDA-MB-435 cell lines and also using MDA-MB-435 cells derived from primary tumor and metastatic lungs in SCID mouse.

#### **3. Preliminary results reported below are for the first RDA experiment (RDA using archival normal and metastatic tissue samples of a DMC patient):**

- i. **Isolation of metastasis associated DNA sequences (MADS):** We isolated normal (glandular and stromal) and metastatic cells (positive lymph node) from a ductal breast carcinoma (RDA-1) using single cell microdissection (Fig. 1). In the first RDA hybridization, the metastatic cell DNA was the tester, and in the second the normal was the tester. As shown in Fig. 2 (a, b and c) the purity of the differential products was

greatly improved from the first round to the third round of hybridization. The 'gain' lane (2) has 4 bands and the 'loss' lane (3) has 5 differential bands (see legend for details). RDA differential products were isolated and DNA was extracted from each of the 5 bands obtained from the loss side of the third round of hybridization and DNA from each band was cloned separately. One hundred clones from each DNA band were saved and a subset of 100 clones (20 clones from each differential product) was selected randomly for further characterization. These 100 clones were probed (labeled with  $^{32}\text{P}$ -dCTP) on dot blots containing normal and metastatic cell DNA. It was found that 79 clones hybridized only with the normal cell DNA, indicating that these sequences were not present in the metastatic cells (Fig. 3a). The remaining hybridized with both normal and the metastatic cell DNA. This may have been due to incomplete subtraction. Of the 79 clones that hybridized only with the DNA of the normal cells, 50 were selected and tested further by Southern blotting to verify the RDA results. None of these hybridized with the DNA of the metastatic cells but all were detected in normal cells (Fig. 3b). Consequently these sequences are candidate losses associated with tumorigenicity/metastasis.

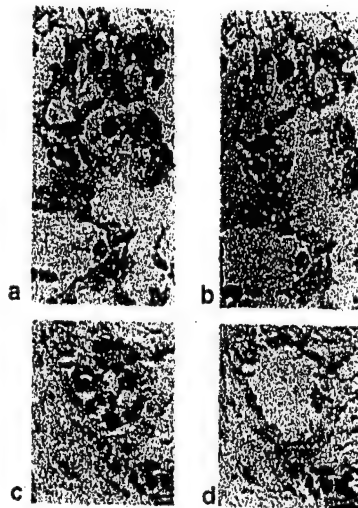


Fig. 1. Single cell microdissection of tumor cells from fresh frozen breast tumor tissues. A tumor cell ( $\Rightarrow$ ) before (a) and after (b) microdissection. The arrows ( $\rightarrow$ ) showing cells for reference and ( $\Leftrightarrow$ ) showing intact collagen material (a) and those floating (b) on the section. Tumor cells in a positive lymph node tissue before (c) and after (d) microdissection.

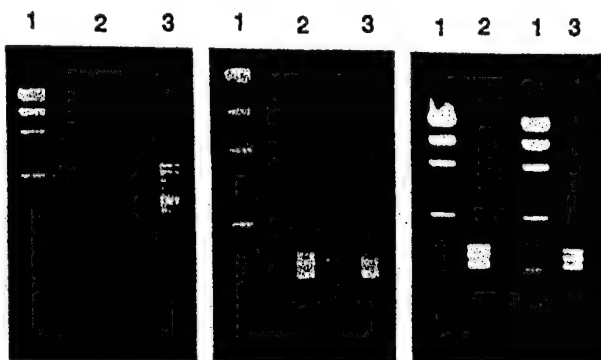


Fig. 2. Isolation of differential products from single cell microdissected archival breast tissue cells using RDA. RDA hybridization was of the normal versus the metastatic tumor cell DNA and was performed in two ways. In the first RDA (Lane 2), the metastatic cell DNA was used as tester (which should yield differential sequences gained during malignant transformation or in the process of becoming metastatic) and in the second RDA (Lane 3), normal DNA was used as tester (which should yield sequences that were lost from the metastatic cells). In the third round of hybridization, the 'gain' lane contained 4 prominent DNA bands ranging from 200-300bp in size whereas in the last lane, there are 5 bands ranging from 200-370bp in size with a prominent band at about 370bp. (a. 1st round; b. 2nd round; c. 3rd round RDA hybridization) Lane 1. Low molecular weight marker.

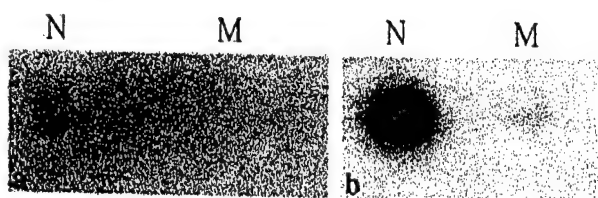


Fig. 3. Results of a typical dot blot and a Southern blot using  $\alpha^{32}\text{P}$ -CTP probes prepared from different groups on normal and metastatic amplicon DNA. a. A positive signal is present only in the normal side but not in the metastatic DNA; b. Southern blot confirming the dot blot results. MADS IX was used as probe in these blotting experiments.



**ii. Sequencing and homology search:** These 50 clones were sequenced and those with identical sequences were grouped. Thus 11 unique candidate metastasis associated DNA sequences (MADS) were identified. The sequence homologies found by searching the gene bank of these MADS are presented in table 1. The search revealed 82-99% homology to known human gene sequences for 9 of these MADSs and one (MADS-IV) was found to be novel, having no homology with the sequences in the gene banks. Previously we reported that we found 2 novel sequences, however last month we found homology to MADS-X in the gene banks. MADS-IX has 94% homology with BAC clone AC022541.10 on chromosome 10. BLAT search of MADS-IX and XI revealed interesting information on the genes and STS markers surrounding these MADS. Within a range of 1.4Mb of MADS-IX we noticed two CDC2 genes (cell division cycle 2 protein, isoform 1 and 2), and two splice variants of ZWINT gene (without known exact function). Besides, in the range of 821Kb, there are 9 STS markers (RH26942, SHGC-58267, SHGC-79901, AFM336XD1, SHGC-103705, SHGC-3856, SHGC-84937, STSG72171, SHGC-81245). MADS-XI has 98% homology with a BAC clone LA009176.1 on chromosome 6. BLAT search of MADS-XI revealed 6 genes (FLJ10975: hypothetical protein, encoding a protein with two PHD-zinc finger domains; FLJ13162: function unknown; DLL1: delta-like 1 protein; PSMB1: proteasome subunit, beta type, 1; TBP: TATA box binding protein; PDCD2: programmed cell death 2) in the range of 1.17Mb and 32 STS markers within 816Kb range. Currently we are screening the patient samples in which MADS-IX and XI were found to be informative, using primers specific to these genes and STS markers, to determine if they are part of the deleted region.

**Table 1.** The primers, the length, homology search and RH mapping results of 11 metastasis associated DNA sequences (MADSs) isolated previously from first RDA experiment. The details about the 4 new MADS are mentioned under 'total probe' (Page# 31)

MADSs	Primers	Length (bp)	Homology (BAC clones)	RH mapping
I	F. ATGCAGGAAGCGCTTGCTTGT R. GGACATCCTCACCATTGATCTC	205	Chromosome 5 (97%) AC005915.1	Not successful
II	F. CGAAGTTCCTGAATCAGTGGGATAT R. GATCTGAATGGTGAGGACGTCAGA	144	Chromosome 21 (99%) AC010463.6	Not successful
III	F. GTGAAGACGACAGAAAGGGCGTG R. GCAGTACCTCTGCAACACTGACG	277	Chromosome 1 (97%) AL359265.8	Not successful
IV	F. ATCTGTGTGTGCAGCGCTGTGGA R. GGAATCTCCGAGGACACTTGAGGT	185	No match	Chromosome 5; Places 19.72 cR from WI-6737
V	F. GGATGTCTTTCCATTTGTTAGGGC R. GGTCTTTCAGATGAATTCACAGATCT	220	Chromosome 16 (98%) AC004234.1	Not successful
VI	F. AAGAGAGGAAATATAGCAGTGGCAC R. GCTTACCGACGCGACTATCCATG	242	Chromosome 7 (97%) AC09333.1	Not successful
VII	F. CAGATAGGTGAGTGTATGTGAAGCA R. ATGGCTGTGCCCTTCTTCCCTA	190	Chromosome 6 (98%) AL121935.17	Chromosome 6; Places 1.51 cR from WI-3110
VIII	F. GCAATGTGGAAGGCATATTTAGAAT R. GTCCACTGGCTGGTAATGGTGGTA	170	Chromosome 6 (82%) AC013429.12	Chromosome 6; Places 3.67 cR from AFMA191WD1
IX	F. AGGTAGGTAGAGTAACAGGTTTGT R. GATCTGTTCTCCCTGGTCTTAGCTT	180	Chromosome 10 (94%) AC022541.10	Chromosome 10; Places 5.66 cR from D10S546
X	F. GATCTTCTCTCTCACAGCTCTGC R. ACCGACGTCGACTATCCATGAACA	192	Chromosome 15 (98%) AC104260.5	Chromosome 15; Places 1.71 cR from D15S157Places 11.09 cR from WI-6813
XI	—	224bp	Chromosome 6 (98%) LA009176.1	Not performed

**iii. Physical mapping of MADS by RH mapping:** To further characterize these MADS we used RH mapping to localize them on human chromosomes. The details of the methodology is described under the Methods section. Based on the sequences of MADS, primers were designed for each group of candidate gene sequences and RH mapping was performed.

The results were arranged as suggested by Research Genetics Inc. (positive bands denoted as 1; negatives denoted as 0 and doubtful ones denoted as 2). The databases at Whitehead Institute/ MIT (<http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper>) were used to obtain the chromosome localization of these sequences. As shown in the Figure 4, those groups that showed expected sized PCR products only in human genomic DNA were subjected to RH mapping (Fig. 5). Of the 10 MADS tested, only 5 (IV, VII, VIII, IX and X) were successfully mapped to specific human chromosomes as shown in Table 1. For MADS VII, VIII, IX and X, the chromosome localizations obtained by physical mapping and by homology search agreed.

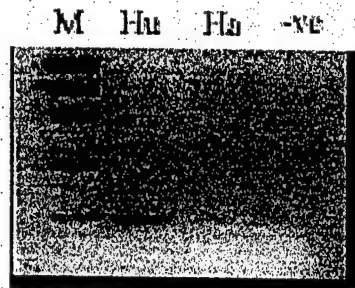


Fig. 4. DNA gel electrophoresis (colors inverted for contrast) showing the presence of expected sizes of PCR products with a MADS-IX in which the template was human DNA and absence of any product in the negative controls (hamster DNA and water).

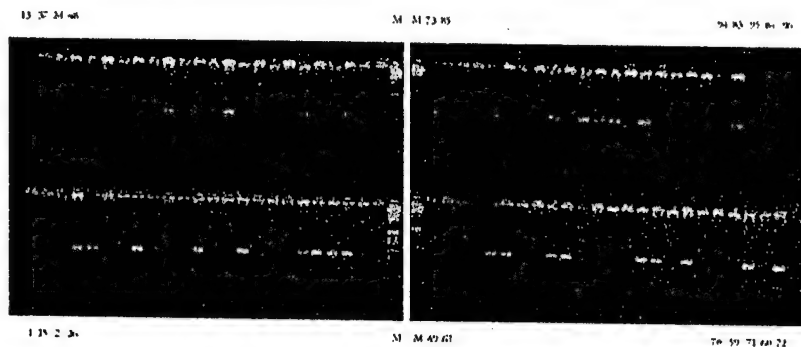


Fig. 5. A typical example of a RH mapping gel (MADS VII). 94 PCRs were run with a pair of primers (MADS-VII) using 94 DNA samples each consisting of a known chromosome segment of human genome. Hamster DNA and water were used as negative controls in the 95<sup>th</sup> and 96<sup>th</sup> PCRs.

**iv. Screening patient samples with MADS-IV:** While screening different MADS on DNA samples of cell lines derived from normal tissue and primary tumors of three breast carcinoma patients who had known losses in specific chromosomal regions, we found that MADS-IV was missing in the tumor DNA samples of one patient cell line (Fig. 6). Since it appears to be homozygously lost in primary tumor cells in PCR results, it must be also lost in metastasis.

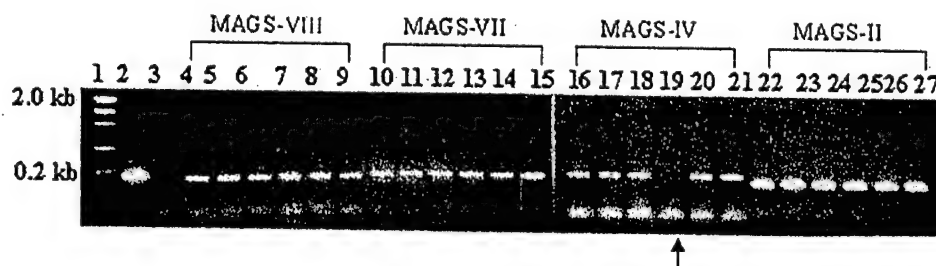


Fig. 6. PCR screening of 4 MADS on DNA samples of paired normal cells (lanes with even numbers from 4-26) and primary tumor cells (lanes with odd numbers starting from 5-27) derived from three breast carcinoma patients with loss of heterozygosity for a region on chromosome 10q encompassing gene PTEN. Lanes 1: 2Kb DNA marker; 2: Positive control; 3: Negative control; PCR results with MADS VIII with product size of 171bp (lanes 4-9); MADS VII with product size of 171bp (lanes 10-15); MADS IV with product size of 190bp (lanes 16-21) and MADS II with product size of 144bp (22-27). Lane 19 represents tumor cell line sample of a patient showing absence of a 185bp product.  $\beta$ -actin internal control is not shown in this figure.



PCR screening of MADS-IV in additional 9 primary tumor DNA samples with matched normal tissue DNA samples, received from Dr. Wahab (Cairo University), we observed the loss of MADS-IV in 2 tumor samples (Tumors 6 and 9). The intensity of the PCR product for MADS-IV was less than half in tumor 6 compared to normal, while it is completely missing in tumor 9, which indicates that MADS-IV probably is heterozygously lost in tumor 6 and homozygously lost in tumor 9 (Fig. 7). However since the DNA samples were not from microdissected tumor cells, it is possible that some other tumor samples (other than 6 and 9) also may have loss of MADS-IV. We therefore requested Dr. Wahab to send primary tumor and the matched positive lymph node tissue paraffin blocks for these 9 cases to isolate tumor cells by LCM for further PCR screening. Dr. Wahab is waiting for approval from the concerned Egyptian and US agencies because of the restrictions due to anthrax related mail problems. Thus far MADS-IV was found to be missing in 3 tumors out of 12 tumors screened so far.

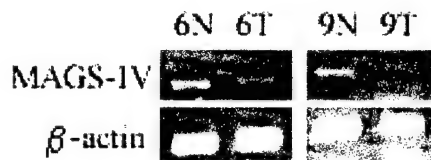


Fig. 7. PCR screening of MADS-IV on normal (N) and tumor (T) DNA samples. Tumor 6 showed heterozygous loss and tumor 9, showed homozygous loss of MADS-IV.

**v. Screening patient samples with MADS-IX:** To determine if these implicated sequences are indeed associated with metastasis rather than transformation, DNA was used from cells recovered with the LCM (Fig. 8) method from normal, primary and metastatic tissue samples from 5 additional patients. These samples were screened by PCR using primers designed from different MADS. As shown in the Figure 9, MADS-IX was present in normal cell DNA samples of all the 5 patients but the intensity of hybridization was less in the primary tumor cell DNA samples from 2 patients and totally undetectable in the metastatic cell DNA of these two patient samples, suggesting a loss of this gene sequence during progression to metastasis. This analysis revealed that MADS-IX was lost in the transition from normal to primary to metastasis in 2 of 5 cases (3 of 6 cases if the case used in the RDA assay is included). The fact that it was present in primary tumor cells, but missing in lymph node metastatic cell foci strongly suggests that this is a marker for a novel metastasis suppressor gene.

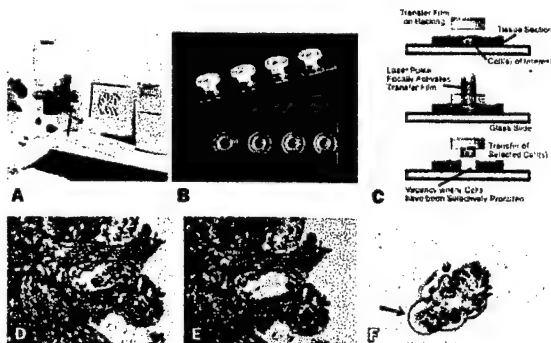


Fig. 8. Laser capture microdissection (LCM) apparatus and illustration of the microdissection procedure. A. The apparatus; B. Membrane caps; C. The principle of LCM method; D. Positive lymph node section before LCM and E. After LCM; F. Dissected pure population of tumor cells.

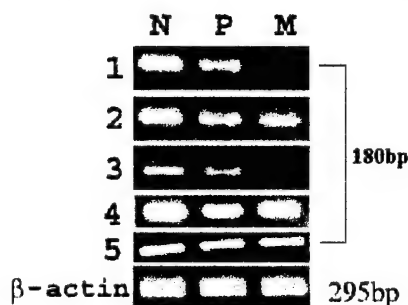


Fig. 9. PCR screening of MADS IX on normal, primary and metastatic cell DNA samples of 5 patients. PCR results showing the target DNA band (180bp) missing in the metastatic cell DNA of patients 1 and 3 (M lane).  $\beta$ -actin as an internal control.

The RH mapping revealed that MADS-IX is localized to a 21cR interval between markers, D105539 and D10S549, corresponding to human chromosome 10 band q21.1 (<http://ncbi.nlm.nih.gov>). To determine if this gene is close to PTEN, we screened 4 tumor cell lines and matched normal DNA (the first 3 tumor cell lines had known losses in specific chromosomal regions, the 4<sup>th</sup> had loss of homozygosity of PTEN gene). PCR screening of MADS-IX and PTEN showed

that MADS-IX is present in all the 4 tumor cell lines (HCC-1806, HCC-1143, HCC-1428 and HCC-1937), especially the 4<sup>th</sup> cell line. PTEN is present in 3 cell lines, but missing in the 4<sup>th</sup> cell line, indicating that it is a novel DNA sequence but neither a part of PTEN gene nor localized in the homozygous loss region of chromosome 10q arm encompassing PTEN region (Fig. 10).



Fig. 10. PCR screening of MADS-IX on normal and tumor DNA samples from breast carcinoma cell lines that losses in specific chromosomal regions (especially the 4<sup>th</sup> which had loss of homozygosity of PTEN gene), showed the presence of MADS-IX in all 4 cell lines. The second row showing the presence of PTEN in the normal cell DNA but missing in the tumor cell DNA of 4<sup>th</sup> cell line (4T lane), indicating that MADS-IX is neither a part of PTEN nor localized in the loss region of 10q which encompasses the PTEN gene.  $\beta$ -actin was used as an internal control.

**Localization of MADS-IX by FISH.** The main aim is to identify a panel of DNA markers that can differentiate the archival tumors which did metastasize from those which did not. Since normal and tumor cells are present side by side in a primary tumor sample, we proposed that FISH is the best way to verify if these markers are present in normal cells and missing (one allele or 2 alleles) in the tumor cells. In these attempts we used MADS-IX as a FISH probe. Generally, FISH requires larger sized probes (DNA fragment: 1-1000kb). Since the size of the MADS-IX is only 180bp, we designed primers from the BAC clone that has homology with MADS-IX, and isolated a DNA fragment sized 2 Kb encompassing the MADS-IX by long-accurate PCR using normal human genomic DNA as the template. This 2 Kb MADS-IX is used as a FISH probe to localize on human metaphase chromosomes to determine that MADS-IX is a human sequence and not an artifact. We labeled MADS-IX with spectrum green (Vysis) and probed on normal human metaphase chromosomes (Fig. 11). The result showed that MADS-IX is localized on chromosome 10 at q21 region (close to centromere on the long arm).

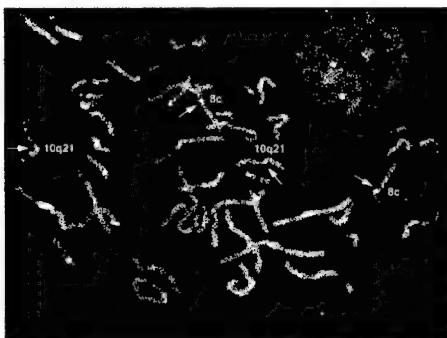


Fig. 11 Localization of MADS-IX on human metaphase chromosomes. Centromere of chromosome 8 was used as a positive control probe. MADS-IX was found to localize around 10q21 chromosome region (close to centromere). Both the MADS-IX and CEP-8 are labeled with spectrum green (Vysis).

**Identification of loss of MADS-IX on a primary tumors by FISH:** Since our long term goal is to screen the MADS as FISH probes on primary tumor tissue sections to predict if that primary tumor is prone to developing metastasis or not, we made an attempt to screen MADS-IX as a FISH probe on normal tissue and primary tumor tissue sections of a patient who developed metastasis. We labeled chromosome 8 centromere (positive control) with orange red and MADS-IX with orange green and screened normal tissue, primary tumor and positive lymph node tissue sections. Normal tissue section showed several red and green signals but the cell/nuclear morphology was not clear due to lot of fat around the cells. In the primary tumor tissue sections, as expected the tumor cell nuclei were much larger than the normal cell nuclei and the FISH signals were clearer than that of the normal cell nuclei. Since we used 2 probes, we expected 3 patterns of labeling signals in the nuclei (presuming the centromere of chromosome 8 is not lost in these tumors), (i): 2 reds and 2 greens, if it is a normal cell nucleus or a tumor cell nucleus that did not lose MADS-IX; (ii): 2 reds and 1 green, if there is heterozygous loss of MADS-IX; (iii): 2 reds and no green, if there is homozygous loss of MADS-IX. As shown in figure 12, we observed clearly all the three patterns in the primary tumor section. Out of 50 good cells observed in the primary tumor section, 26 clearly were of first pattern (normal), 20 were of second pattern and 3 of third pattern. Similar FISH in the

positive lymph node tumor section of the same primary tumor showed more pattern 3 cells rather than pattern 2 cells. Recently we screened another primary tumor that did not develop metastasis. Out of 50 cells observed, interestingly we did not record any cell that belonged to pattern 3 and only 3 cells that showed pattern 2 (loss of heterozygosity). These results are preliminary but they are promising. As mentioned elsewhere in the present FISH preparations, we switched the dyes to avoid confusion between the spectrum green labeled target sequence signal and artifact green color signals generated by fat lobules in the primary tumor section. In the present grant application we proposed to FISH screen all the 15 MADS (Hopefully we will be able to find homology to MADS-IV in the gene banks soon. Otherwise we will approach Celera to provide the homology information) on thinner (4-5um) tissue sections with better cell morphology from breast carcinoma primary tumor tissue sections consisting of 40 cases that did and 40 cases that did not metastasize, in the first aim. We also proposed to transfer cells from tumor section onto the slide by just touching the slide surface. In this way we will be able to observe very clean preparations without any overlapping of cells from the tumor section which also facilitates recording of FISH results without confusion.

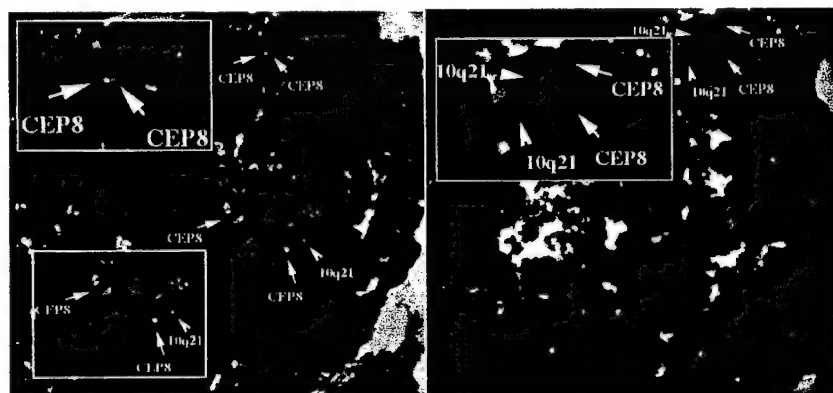


Fig. 12. Localization of MADS-IX in different cells of primary tumor tissue section. Chromosome 8 centromere (positive control with red signals) was labeled with orange red and MADS-IX with orange green (green signals). 3 patterns of labeling signals in the nuclei were observed: (i): 2 reds and 2 greens, if it is a normal cell nucleus or a tumor cell nucleus (right side top inset) that did not lose MADS-IX; (ii): 2 reds and 1 green (left lower inset), if there is heterozygous loss of MADS-IX; (iii): 2 reds and no green, if there is homozygous loss of MADS-IX (left top inset).

**vi. Screening patient samples with MADS-XI:** The MADS-XI, has an interesting and also novel pattern of TG/AC repeats (Fig. 13). Since dinucleotide repeats (TG/AC) are correlated in several cancers with the severity of the disease, we became interested to screen some breast cancer patient samples. PCR screening of patient samples was not possible due to TG repeats. Consequently we used the sequence as a  $\alpha^{32}\text{p}$ -dCTP probe to screen a dot/Southern blot consisting of normal, primary and metastatic cell DNA of 5 patient samples (C-14153; C-1050; DS 97-919; C-14852; DS-711). The primary and metastatic tumor cells were isolated by LCM. The results showed that MADS-XI was missing in 4 out of 5 patient samples (Fig. 14). Analysis of additional patient samples using MADS-XI is underway.

**VGATCTTAATCCGGGGAGTGGCGTATGTAGTAGAAGAGTCTGGATTTGAGTAGTGTATGGT**  
**AACGCCAG** (T-G)<sub>5</sub> C (T-G)<sub>4</sub> GTA (T-G)<sub>2</sub> G (T-G)<sub>4</sub> T (T-G)<sub>3</sub> GCA (T-G)<sub>3</sub> G (T-G)<sub>2</sub> TATGG (T-G)<sub>3</sub> G (T-G)<sub>3</sub> A  
 (T-G)<sub>4</sub> GTGCG (T-G)<sub>2</sub> AGATACGTGG (T-G)<sub>3</sub> GGG (T-G)<sub>6</sub> G (T-G)<sub>3</sub> G (T-G)<sub>5</sub> GTA (T-G)<sub>2</sub> G (T-G)<sub>3</sub> G V.

Fig. 13. Complete MADS-XI with unique sequence (bold letters) and enriched with TG repeats; V= vector sequence

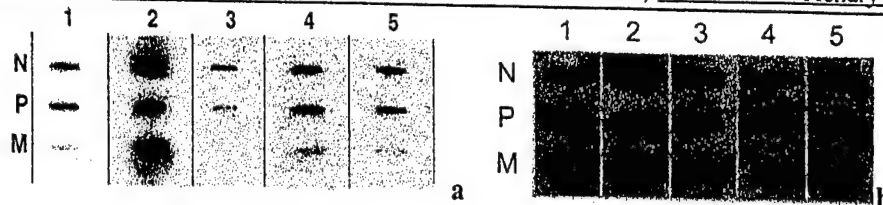


Fig. 14. a. Results of slot blots using  $^{32}\text{P}$ -CTP probes prepared from MADS-XI on normal (N), primary (P) and metastatic (M) genomic DNA samples from 5 ductal breast carcinoma patients. In cases 1, 3, 4, and 5, MADS-IV hybridized to normal and primary but not to metastatic cell DNA, probably indicating the loss of this sequence in metastatic cell DNA of these cases. Case 2 however showed signals in N/P/M samples. b. Ubiquitin gene was used as an internal control.

**vii. Further characterization of MADS:** To determine if a MADS is an expressed sequence of a gene and if expressed, is it tissue specific (expressed only in breast tissue) or not, we performed RT-PCR and normal human mRNA dot/Northern blotting experiments using the MADSs. For RT-PCR experiments, primers were designed from different MADS and used on normal human total RNA. RT-PCR results showed the expected size products of MADS-II and -IV, indicating that they are expressed sequences (Fig. 15).

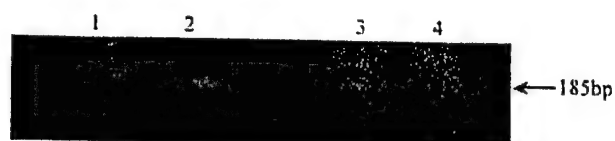


Fig. 15. RT-PCR of normal RNA using MADS-IV primers showing that the gene is expressing. Lane M: 200bp DNA marker band; Lane 1: Positive control; Lane 2: Negative control; 3-4: Total RNA from normal human tissue digested with DNase I.

With regard to homology search no matches were found for MADS-IV in the gene banks indicating that it is possibly a novel DNA sequence associated with breast cancer. RT-PCR results showed that MADS-IV is an expressed sequence. To confirm this, we screened multiple tissue RNA Master Blots<sup>TM</sup> (CLONTECH Laboratories, Inc.) using MADS-IV as a  $\alpha^{32}\text{p}$ -dCTP probe. Results revealed that MADS-IV expresses in mammary gland and also in all the other human tissues, but not in non-human RNA or DNA samples (*E. coli* synthetic poly r(A), yeast tRNA or total RNA, or *C<sub>0</sub>t1* DNA), indicating that MADS-IV is a part of a human gene that transcribes commonly in human tissues (Fig. 16). This reiterates the fact that this MADS is part of a functional gene possibly involved in breast tumorigenesis/metastasis. Full Northern blots are being performed to confirm these results and also to determine the actual size of transcript of this gene.

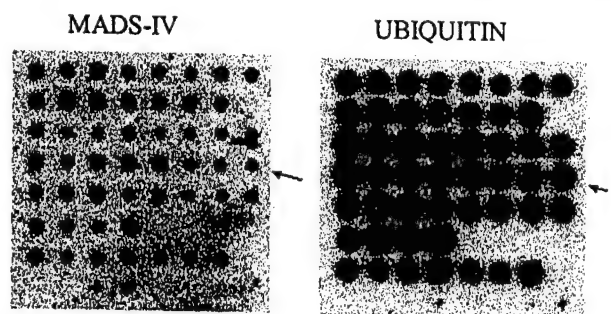


Fig. 11. Results of Northern hybridization of RNA Master Blot (CLONTECH Laboratories, Inc.) using MADS-IV as probe. It showed expression in all of the human tissues, but not in non human RNA or cDNA (synthetic poly r(A), yeast tRNA or total RNA, or *C<sub>0</sub>t1* DNA) indicating that it is an expressed human gene sequence. No significant change in the intensity of signal was noticed in breast tissue (Arrow) compared to the remaining tissues. Ubiquitin gene was used as an internal control for normalization.

**viii. Isolation of complete MADS:** Generally RDA recovered size of differential sequences are in the range of 100-350bp. For example, MADS-IV is only 185bp. Since MADS-IV seemed to be a novel sequence without any homology in the gene banks, in order to obtain partial/full-length sequences of MADS-IV, we used human mammary cDNA library screening and Inverse-PCR (I-PCR) methods. The reason behind these 2 approaches is to obtain complete gene sequence or reasonably long enough sequence so that we can use these MADS for transfection studies as well as use as FISH probes (more than 1Kb size of DNA sequence is required to use as FISH probe) to screen normal and tumor cells simultaneously on primary and metastatic tumor tissue sections of patients (Groups I and II). However recently we changed our approach in this regard. We are planning to use BACs containing MADS directly as FISH probes and also transfect retrofitted BACs containing MADS into highly metastatic human breast carcinoma cell lines to evaluate their metastatic potential.



**4. Other RDA experiments:****i. Isolation of MADS by RDA using archival primary and metastatic breast carcinoma cells:**

Our aim in this project is to isolate primary and metastatic cells from archival breast tissue sections and subject their DNA to RDA to obtain genetic markers involved in the transition from primary to metastasis. We therefore isolated cells from the primary and metastatic tissues of 8 patients using laser capture microdissection equipment (Arcturus Co.) available in our LCM core facility at AECOM (Fig. 8). In the first RDA hybridization, the metastatic cell DNA was the tester and in the second, the primary was the tester. DNA was extracted from the DNA bands on the loss and gain sides and cloned using TA cloning system (pCR2.1; Invitrogen Co.). One hundred clones from each of the 8 RDA (loss) experiments were collected.

**ii. Isolation of MADS by RDA using mammary carcinoma cell lines:** We subjected a pair of cell lines derived from MDA-MB-435 to genomic RDA (GRDA). One cell line, HI-177, was low metastatic and other one, C-100 was highly metastatic. The other pair of human mammary carcinoma cell lines were derived from primary tumors and the corresponding metastatic lungs in SCID mice (Price, 1996). Similar to other RDA experiments differential products from loss and gain were cloned and randomly 50 clones from each side were collected from RDA of both paired cell lines.

**5. Total Probe:** From each of the RDA experiments (loss side) except for the first experiment, 50 clones were selected and transferred onto separate nylon membranes. In this way we prepared 8 membranes for 8 RDA experiments using tissue samples and 4 membranes using the cell lines. The DNA from the 11 MADS (isolated from the first RDA) were mixed in equal amounts and used as a 32p-dCTP probe (Total probe) on each of the 12 membranes. We picked up 47 clones that did not hybridize with the total probe. We believe that these sequences are novel (not present in the 11 MADS mixture). Similar to those differential sequences that were isolated from the first RDA, these 47 candidate clones were sequenced. Sequencing revealed additional 4 groups of MADS. Thus bringing the total number of MADS to 15.

The 4 new MADS have the following homology in the gene banks:

MADS-XII: Product size: 125bp; 96% homology with BAC clone RP11-651C2 on chromosome 4.

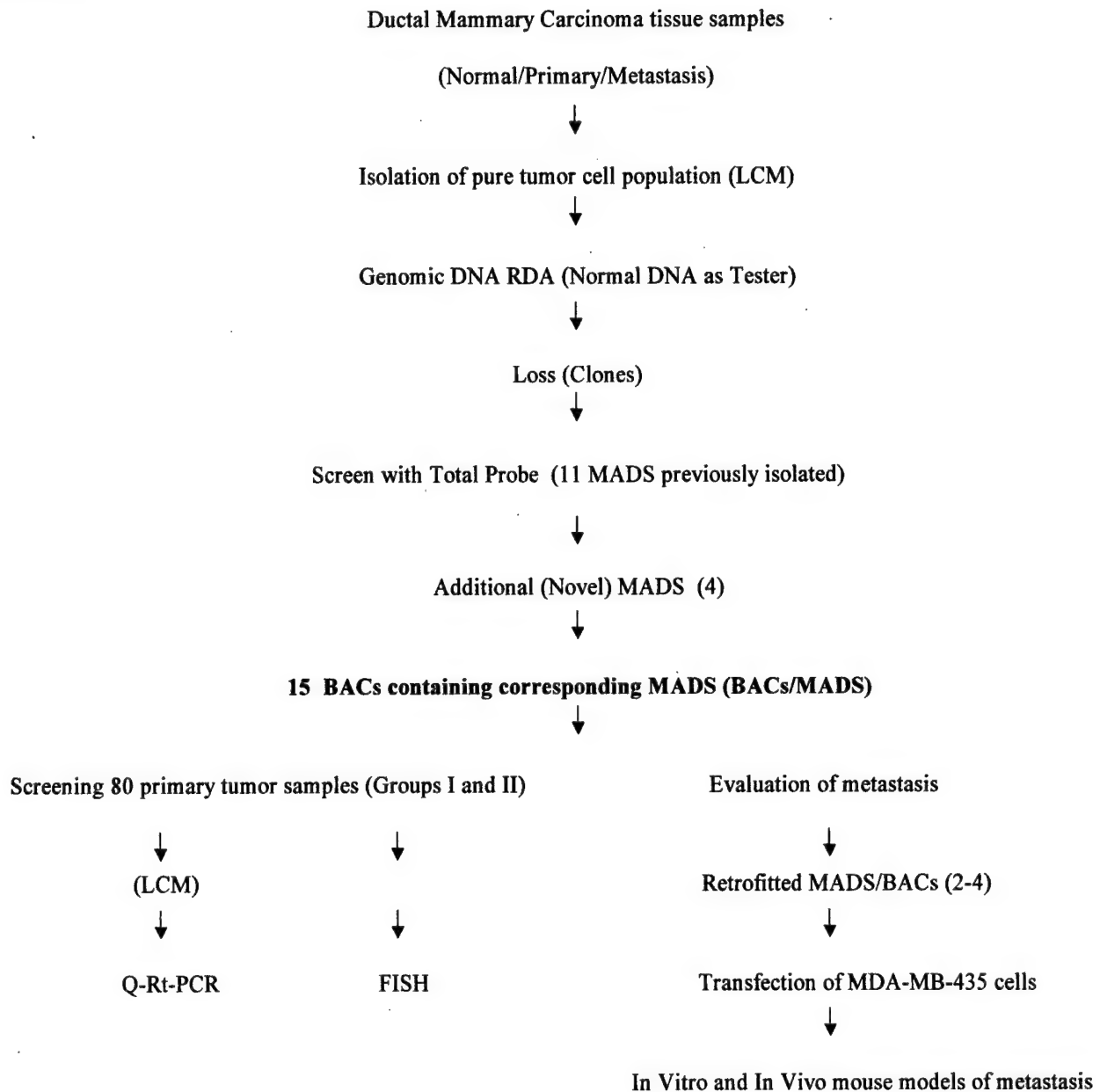
MADS-XIII: Product size: 212bp; 99% homology with BAC clone RP11-452C13 on chromosome 7.

MADS-XIV: Product size: 231bp; 92% homology with BAC clone AC000119 on chromosome 7.

MADS-XV: Product size: 172bp; 95% homology with BAC clone AL590825 on chromosome 6.

**PI background information:** The P.I. of this application, Dr. Achary, has a Ph. D. in Molecular Cytogenetics. During his Ph. D. training he worked on different aspects of DNA replication in *Drosophila* using chromosome and DNA fiber autoradiographic techniques. He then spent one year in post-doctoral training in molecular biology at the NIH/NIDDK in 1994-95. He has been working as a Research Associate in the Department of Radiation Oncology at Albert Einstein College of Medicine (AECOM) since August 1994 and was promoted to Assistant Professor in July 1999. During this period he acquired additional experience in the basic methods of molecular biology including the more specialized techniques of RDA, RH mapping, LCM, and the cDNA microarray methods. Dr. Achary was trained in the laser capture microdissection method by Dr. Z. Zhuang and M. Emmert-Buck of the Developmental Molecular Diagnostic Unit, Laboratory of Pathology, NCI-NIH, Bethesda, MD. He has been working closely with Dr. Kucherlapati, the previous chairman of the Department of Molecular Genetics and the director of Microarray facility at Einstein and has been collaborating with Dr. Harold P. Klinger, Professor of Molecular Genetics and Pediatrics at AECOM for these past three years as well as with other eminent molecular geneticists and tumor biologists at Albert Einstein College of Medicine and its NIH/NCI supported Cancer Research Center. He has a three-year Idea grant from the US Army and a pilot grant from RTOG. These funds have allowed Dr. Achary to set-up a facility for undertaking cDNA microarray studies in the Department of Radiation Oncology in collaboration with the Departments of Molecular Genetics, Pathology and very recently Molecular Pharmacology. Besides looking for DNA markers of breast metastasis his group is also using cDNA microarray and proteomics methods to identify prognostic markers of breast and cervical cancers. They have been using nm23 (Metastasis suppressor) and erbB2 (Metastasis promoter) transfected human mammary carcinoma cell lines (MDA-MB-435) and the resulted tumors in SCID mouse model to understand the signal transduction pathways of metastasis mediated by nm23 and EGFR family members especially ErbB2. Dr. Achary is an active member of RTOG and served on the RTOG cervical tumor tissue committee along with Dr. Carlo Croce, Director of Jefferson Cancer Institute, Philadelphia and Dr. Sally Amundson of NCI/NIH in 1999. Dr. Eliot Rosen of LIJ, Dr. Elias Lianos of UMDNJ, Dr. Jeff Segall of AECOM and Dr. Giulio Maria Pasinetti of Mount Sinai Medical Center have been collaborating with the PI for

their cDNA microarray studies. He is presently being trained at Mount Sinai Medical center in the use of proteomics in breast cancers. Several labs at AECOM, UMDNJ and Kansas Cancer Center collaborate with the PI for his expertise in LCM. Dr. Achary has been strongly motivated by the environment at Einstein, and the ongoing research work, have stimulated a strong interest for tumor biology, particularly in cancer diagnostics, an area to which he intends to devote his career. It is expected that the experience gained from the ongoing work and the knowledge accumulated from participation in journal clubs, seminars, laboratory conferences and many other activities of the Cancer Center, the Genome Center, the Departments of Molecular Genetics and Radiation Oncology, will catalyze the applicant's development and lead to his becoming a productive cancer investigator.

**Research design:**



**Specific Aim 1:** To isolate a panel of DNA sequences for detecting primary tumors those did (Group-I) and those (Group-II) did not develop metastasis.

As reported in the preliminary data, we have previously isolated 11 metastasis associated DNA sequences (MADS) and three of these were shown to be missing in samples of metastasis from additional patients. From subsequent 10 RDA experiments using the 'total probe' method we have identified 4 additional MADS. We propose to screen these 15 candidate MADSs step-wise by a quantitative real-time PCR (Q-Rt-PCR) and by fluorescence in situ hybridization (FISH) methods on primary tumors of 40 patients who developed metastases in the lymph nodes (Group-I) and 40 patients who did not develop metastases in the lymph nodes within 5 years of primary tumor detection (Group-II). Tumor cells will be isolated by Laser Capture Microdissection (LCM) for Q-Rt-PCR analysis. We have designed primers for all the MADSs for Q-Rt-PCR and also identified BACs that contain respective MADSs and also currently using the whole BACs as FISH probes.

**Experimental steps:**

1. The first step is for the collaborating pathologist, Dr. Jones to reconfirm the clinical parameters of the 80 ductal mammary primary tumors and 40 positive lymph node samples corresponding to the 40 primary tumors that developed metastasis (Group-I). All the tumor blocks will be coded by Dr. Jones and provided to the PI (blinded).
2. \*Cut about 20 tissue sections (4-5um) from each of the 80 primary tumors and 20 sections from each of the 40 positive lymph nodes. One will be used as a reference slide with H & E staining, 15 sections for FISH, 2 for LCM and 2 saved as back up.
3. Isolate tumor cells from 80 tumors (2 slides from each) by LCM for Q-Rt-PCR analyses of 15 MADS.
4. Confirm the presence of the MADS in respective BACS by PCR using primers designed from the MADS.
5. Q-Rt-PCR analyses of 15 MADS on DNA samples derived from 80 tumor samples (Step 3).
6. Use BACs containing MADS as FISH probes on tumor tissue sections of 80 primary tumor samples.
7. Q-Rt-PCR and FISH screening on 40 positive lymph node samples.
8. Determine the consistency between Q-Rt-PCR and FISH results in 80 primary tumor samples.
9. Decode the blinded samples and determine if groups-I and II could be differentiated by screening of 15 MADS with Q-Rt-PCR or FISH or both methods.

\*Attempts will be made simultaneously to use a simple method to prepare slides with tumor cells without using cut tissue sections from the tumor block. The tumor tissue material is exposed by trimming of the paraffin. Material from the block and then by touching the tumor tissue side at several places on the slide which will allow some cells to attach from the block on to the slide surface. Conventional methods will be used to fix the cells followed by standard FISH procedure. We have not yet used this method in PI's laboratory. However PI has watched Dr. Zhuang's group using this method at NCI for FISH and fibre FISH analyses.

**Expected results and potential pitfalls:**

The techniques involved in this proposal (LCM, RDA, Q-Rt-PCR, FISH etc.) are robust and hold great promise to achieve the objective in this grant application. Based on our preliminary results, we expect that the first aim will yield a panel of useful DNA markers which could differentiate primary tumors that did develop metastasis from those which did not develop metastasis. As shown in the table 2, we expect that a significant number of tumors in group-I will show loss of heterozygosity for majority of MADS and the corresponding positive lymph nodes should show homozygous loss. Similarly a significant number of tumors of group-II will show no loss for those MADS. However marker status comparisons will be made only between group-I primary tumors versus Group-II primary tumors. In FISH screening the target BAC/MADS will be labeled with Spectrum orange (previously we labeled with spectrum green and green signals were sometimes difficult to identify if fat lobules which take lighter green stain are present in the tissue section) and the control probe (centromere of chromosome 8) will be labeled with spectrum green or some other dye. In such a situation, normal cells will have 2 red and 2 green signals; cells with heterozygous loss of target MADS will have 1 red and 2 greens and cells with homozygous loss will have only 2 green signals. Q-Rt-PCR results, FISH results along with tumor data will be tabulated for analysis.

**Table. 2.** Hypothetical status of DNA markers (For example, 5 markers, I-V) in groups I (For example, 5 tumors) and II (For example, 5 tumors) breast carcinoma tumors. No loss= ++; Heterozygous loss= +/-; Homozygous loss= -/-

Group-I tumors	1	2	3	4	5	Group-I +ve L. Nodes	1	2	3	4	5	Group-II tumors	1	2	3	4	5
MADS-I	+/-	+/-	+/+	+/-	+/-	MADS-I	-/-	-/-	-/-	-/-	+/-	MADS-I	+	+	+	+	+
MADS-II	+/-	+/-	+/-	+/-	+/-	MADS-II	-/-	-/-	+/-	-/-	-/-	MADS-II	-	+	+	+	+/-
MADS-III	+/+	+/-	-/+	+/+	+/-	MADS-III	+/-	-/-	-/-	-/-	-/-	MADS-III	+/-	+	+/-	+	+
MADS-IV	+/-	+/-	+/-	+/-	+/-	MADS-IV	+/-	-/-	-/-	-/-	-/-	MADS-IV	+	+	+	+	+
MADS-V	+/-	+/-	+/+	+/-	+/-	MADS-V	-/-	-/-	-/-	+/-	-/-	MADS-V	+	+	+	+	+

1. As mentioned in the preliminary results, we used techniques like inverse-PCR, Genome walker and Long & Accurate PCR to generate long sequences from the intronic sequences (MADS) that were generated by RDA. Similarly for exonic sequences (MADS) we used cDNA and BAC library screening methods. Subsequently we generated intronic DNA sequences (MADS-I and -IX) long enough (2Kb) to use as FISH probes. We also isolated few positive cDNA clones from a breast carcinoma cDNA library from an exonic sequence, MADS-IV. These clones are being characterized. However, we will not continue to use these techniques to isolate long DNA sequences or full-length cDNA sequences in the present proposal. Instead, as mentioned elsewhere we will directly use the BACs containing the MADS as probes in FISH screening methods. We previously expected that the size of the BAC insert may be larger than the deletion. Therefore we presumed that the large BAC may hybridize to the either side of the extended sequence of the deletion leading to misinterpretations of results. However after consultation with several Cytogeneticists now we realize that most of the deletions are larger than the BACs, therefore we proposed to use directly BACS as FISH probes in specific aim 1. In any case the FISH results should corroborate with Q-Rt-PCR results.

2. Q-Rt-PCR and FISH methods are working very well in our hands and we expect that the results of one will strengthen the results of the other in the present investigation. We expect to find a unique but consistent pattern of presence/absence of the 15 MADS in majority of the primary tumors that developed metastasis (Group-I) which will be different from that of Group-II primary tumors. At this stage Drs. Negassa, Jones and Klinger and PI will analyze these data with respect to the clinical parameters of the tumor samples to determine if a statistically significant panel of markers could be established to reliably identify ductal mammary carcinomas that are prone to developing metastases (Group-I) from those that are not likely to metastasize (Group-II). Statistical considerations were mentioned in methods section and a letter from Dr. Negassa is enclosed.

3. The advantage of RDA over other subtraction methods is the "representation" step which lowers the DNA complexity of both tester and driver. This allows one to achieve greater completeness during subtractive enrichment and hence a more effective kinetic enrichment. Similar to most PCR based techniques, however, the RDA method has a considerable level of background, leading to concerns about the success of isolating genes in breast tumor progression. However, the experience of others with RDA in other types of tumors (Li et al., 1997) and our preliminary studies support this approach for identifying DNA maker sequences and possibly new genes

4. Another concern is that RDA may not be productive to compare primary tumor and metastatic cells, to find MADS, since many of the changes identified may occur subsequent to metastasis but not be the cause of metastasis. Furthermore, in order to metastasize, cells within primary tumor must have alterations in the relevant gene sequence. Therefore RDA may fail to detect a difference if the loss is present in both primary and metastatic cell types. Though this is possible, we strongly believe that the current strategy will be effective because our primary tumor cell population should include a large percentage of cells that have not lost the gene and are not destined to become metastatic. Therefore a difference can be detected. When normal cell DNA from a subject was used both as driver and tester in RDA it gave no differential products indicating that the sequences hybridized were similar, thereby total subtraction after three rounds of hybridization (personal communication from Lisitsyn, N.). Based on this information, when we compared the microdissected cells from primary and metastatic cells, if there was no difference between them with regard to their genetic make-up, then we should not have gotten differential bands after three rounds of hybridization. As mentioned elsewhere we performed successfully 8 such RDA experiments that yielded several differential products. Our FISH results using MADS-IX on primary tumor tissue sections support this argument. Besides, referring to the preliminary results section, we believe that this approach can be successful. For example, when the MADS-XI was hybridized on the normal, primary and metastatic cell DNA,

signal was found to be missing (at least 4 out of 5 cases) only in metastasis but not in normal and primary cell DNA. Similarly MADS-IX was found to be missing in the metastatic cell DNA of 2 out of 5 patient samples indicating that we are able to isolate those sequences that are lost in metastasis. Besides, a number of useful discoveries have been made using this technique including PTEN and DLC-1, a novel gene, frequently deleted in human liver cancer (Yuan et al. 1998). We should be able to discriminate what are likely to be etiologically important sequence changes from random changes of no or little importance, by the fact that changes relevant to the metastatic process would be expected to be found in a large population of different mammary tumors with similar histopathologic and clinical parameters.

5. PCR screening of the MADS using microdissected tumor cell DNA from different samples may not provide consistent results. For example in one of our Southern blots a MADS was found to be present in normal and was missing in metastatic tumor cell DNA. However PCR results from the same samples showed that the MAG was found in both normal and metastatic cell DNA. This indicates that even if we carefully perform microdissection (LCM/SCM), contamination of even few stromal cells in a large population of tumor cells may provide misleading results for genes lost in metastasis. On the other hand, Southern blot is a robust technique but requires larger quantities of DNA. Biopsy samples, however, provide limited DNA. To circumvent this problem we use the amplification method proposed for 'representation step' in RDA experiments (Lisitsyn et al 1993) for the blotting experiments. Instead of ordinary PCR we therefore started using Q-Rt-PCR. However we believe that FISH probes will provide more detailed information as the probes will be hybridized on tumor tissue sections consisting of tumor cells surrounded by stromal cells. We expect that the MADS should be present in normal cells and missing in metastatic tumor cells in the same tissue section. By examining primary tumor sections with FISH we should be able to identify the subpopulation of tumor cells that have loss and may be likely to metastasize. FISH may help identify a subpopulation of primary tumor cells that have lost those MADS that are a prerequisite for metastasis.

**Specific Aim 2:** To determine if BACs containing MADS have inhibitory effect of metastasis. Highly metastatic MDA-MB-435 human mammary carcinoma cells will be transfected with the retrofitted BAC containing MADS of interest. *In vitro* and also *in vivo* mammary SCID mouse models will be used to determine if the highly metastatic cell line transfected with the BAC/MADS changes to low or non-metastatic phenotype. \*\*Most promising MADS (2-4; 1-2 per year) will be selected for functional studies.

#### Experimental steps:

1. Construction of retrofitted BACs containing \*\*most promising MADS (BACs/MADS) with selectable marker.  
\*\*Most promising MADS will be selected basing on the following criteria:
  - i. If a MADS is missing (heterozygous loss) in a significant number of primary tumors and completely missing (homozygous loss) in the corresponding positive lymph nodes
  - ii. If BLAT search results indicate that genes associated with tumorigenesis or metastasis are located within the deleted fragment (BAC insert)
  - iii. If Blast search results show high similarity with any genes with cancer related functions.
2. Transfection of retrofitted BACs/MADS into MDA-MB-435 metastatic human mammary carcinoma cells.
3. Isolation of MDA-MB-435 cell clones containing BAC/MADS by selectable marker screening.
4. Evaluation of the metastatic potential of the BAC/MADS transfected MDA-MD-435 cells using In Vitro (Cell invasive assay) and In Vivo methods (Spontaneous mammary fat pad mouse model).
5. Identification of BACs containing putative metastasis suppressor genes.

#### **Expected results and potential pitfalls:**

We expect that some of the BACS containing MADS will show metastasis inhibitory potential. This will then indicate that at least one metastasis suppressor gene (MAG) may be residing in the BAC insert in proximity to the corresponding MADS. However isolation and further functional studies of the putative MAG will require a separate grant application.

1. In Vitro methods are mostly limited to invasion studies using matrigels and may not really reflect the metastatic potential of the transfectant cells. So we added an In Vivo mouse model experiment to evaluate the metastatic inhibitory potential of BAC/MADS.

2. BAC libraries are widely used at present because they are commercially available and they have the advantage of containing large inserts that average 140kb (Shizuya et al. 1992). In addition, BACs are very stable because they are based on the *Escherichia coli* F-factor plasmid, which maintains a very low copy number in bacterial cells, thus minimizing the possibility of recombination and resultant chimeric clones. In spite of these advantages, BACs cannot be used as shuttle vectors because they do not contain a selection system or reporter genes suitable for expression in eukaryotic cell lines. Thus, when BACs of interest are identified, it is necessary to modify or "retrofit" them to use them for biological studies. Current methods of retrofitting BACs rely on performing restriction enzyme digests to isolate genomic fragments, followed by ligation into vectors that contain the desired markers (Mejia and Monaco 1997) or on homologous recombination with a shuttle vector that must be constructed specifically, based on the sequences present in the genomic insert (Yang et al. 1997). We believe that this retrofitting strategy using RETRObac will prove to be a valuable resource for isolating and analyzing new genes, as it allows for direct selection of cells that contain transfected BACs. The large size of the genomic insert in the BAC vector improves the chances that a single BAC clone will contain a gene in its entirety, along with its regulatory regions, making them well suited for eukaryotic genetic transfer studies (Kim et al., 1998).

3. We have transfected a metastasis suppressor (nm23) gene and a metastasis promotor (ErbB2) gene with plxn vectors into MDA-MB-435 cells and successfully examined the primary tumors, lymph nodes and lungs in a SCID mouse model. So we can easily transfect BAC/MADS into 435 cells. We have not done retrofitting of BACs in our laboratory. In this regard Dr. Athwal of Temple University, agreed to help us if we face any difficulty in the construction of retrofitted BAC/MADS.

4. Transfection experiments will give a functional readout. If the effect is not +/- then quantitative effects will need to be evaluated using a large enough series of animals to detect a statistically significant difference. Histologic imaging techniques will be employed to quantitate the number and measure the size of metastatic foci in the lymph nodes as well as lungs. Verification of functional relationship between expression of gene(s) and decrease in metastasis will support a future study proposal to investigate function in mice with targeted knock out of the gene in tumor cells. Since retrofitted BACs containing the MADS are used, no matter if the MADS are expressed or unexpressed sequences. In either case the metastatic potential could be tested in this format. However, those that can be tested and which show an in vivo effect, will be valuable in isolating novel genes associated with metastasis.

#### **Time Table:**

Task 1. Specific aim 1 : Characterization of additional RDA products	1-4 months
Task 2. Specific aim 1 : Sectioning of 80 tumor samples	1-3 months
Task 3. Specific aim 1 : LCM of 80 tumor samples	3-8 months
Task 4. Specific aim 1 : Q-Rt-PCR of 80 tumor samples	3-14 months
Task 5. Specific aim 1 : FISH of 80 tumor samples	3-18 months
Task 6. Specific aim 2 : Preparation of retrofitted BACs containing MADS	8-12 months
Task 7. Specific aim 2 : In vitro metastasis evaluation of MADS	8-12 months
Task 8. Specific aim 2 : In vivo metastasis evaluation of MADS	8-20 months
Task 9. Statistical analysis of complete results (Co-relation of MADS to patient groups I and II)	20-22 months
Task 10. Preparation of manuscripts and final grant report	22-24 months

#### **Methods (I):**

(Being used/to be used in the experiments proposed in this revised application)

#### **1. Archival DMC tissue samples:**

Thus far, we have collected about 120 ductal mammary carcinoma (DMC) which have at least 5 years of follow up. These archival ductal mammary carcinomas (DMC) were collected from the Co-operative Human Tissue Network, Pathology Department of AECOM/MMC and NCI/NIH. From the clinical records Drs. Jones and Klinger found that out of 120 cases, 50 primary tumors have developed metastasis and remaining 70 have not developed metastasis. For this investigation we have selected 40 primaries that developed lymph node metastasis and 40 primaries that did not develop lymph node metastasis within 5 years of cancer detection. Recently paired DNA samples from normal tissue and primary tumor of 9 DMC were received as gift from Dr. A. Wahab of Cairo University. He will be sending soon a set of 100



primary tumor samples (with positive and lymph nodes) and matched normal DNA with all the available clinical parameters (His letter enclosed). If time permits we will extend our studies to these samples. To generate preliminary data so far we have used 9 cases of ductal mammary carcinoma tumors, measuring less than 2 cm that have metastasized to lymph nodes. These tumors are characterized histopathologically as, well (3 cases), moderately (3 cases) and poorly (3 cases) differentiated. The already generated data and the data that will be produced during this study by PCR and FISH screening of 15 MADS, the clinical parameters of 80 patients will be entered into a separate computerized database we have established to facilitate later analysis of the various tumor parameters. Appropriate statistical analyses of the data will be performed by Dr. Negassa, a biostatistician in the Department of Epidemiology and Social Medicine at AECOM (His letter is attached). Based on the preliminary results, he suggested the following statistical consideration:

#### **Statistical considerations:**

We have isolated about 11 candidate metastasis associated DNA sequences (MADS) that were found to be lost in metastatic cells. When screened on normal, primary and metastatic cell DNA samples from 10 breast carcinoma patients one of them (MADS-XI) was found to be lost in the metastatic cells of 3 patients and another sequence (MADS-XI) was found to be lost in 2 patients. This suggests an overall 50% genetic alteration with a 95% confidence interval of (20%, 80%) derived from a non-parametric bootstrap (Efron and Tibshirani, 1993). The wide confidence interval is attributed to the small size of the preliminary data. Assuming that the underlying percentage of genetic alterations is about 13%, i.e., same as the percent of mammary (lymph node negative) carcinomas that are prone to metastasize in lymph nodes, a sample size of 80 will enable us estimation within  $\pm 0.07$  precision with 95% confidence level.

In total 80 archival tissue samples will be used to correlate these genetic markers, retrospectively, with clinical outcome of patients. We will employ logistic regression (Hosmer and Lemeshow, 1989) in order to assess the association between these genetic markers and clinical outcomes (i.e., metastasis and recurrence). The analysis will be adjusted for established prognostic factors such as lymph node status, ER/PR status, Her2/neu oncogene and Lympho-vascular invasion (Roses, 1999). Given the aims of the study and the number of subjects to be involved, it is not realistic to consider all possible prognostic factors in the multivariable analysis. It is well known that most breast cancer prognostic factors are correlated with each other (Roses, 1999), therefore, restricting ourselves to the major risk factors is a reasonable approach and this also minimizes the problem of multi-collinearity during analysis. The results of the analysis will be reported as odds ratios with associated 95% confidence intervals. Since this is an observational study with a relatively small number of study subjects, there is a possibility of facing sparseness while attempting to look at a combination of prognostic factors simultaneously. In such circumstances, we will apply Exact approaches for binary data analysis (Mehta et al. 1992), and will report odds ratios and 95% Exact confidence intervals.

#### **2. Isolation of cells from biopsy samples by laser capture microdissection (LCM).**

This microdissection (Emmert-Buck et al., 1996) allows efficient recovery of a large number of cells and minimizes contamination by non tumor cells from the archival histologic sections. This procedure thus obviated the use of labor intensive single cell microdissection method. LCM (Emmert-Buck et al, 1996) was used for isolating tumor cells from primary and metastatic tissue samples of additional patients for screening candidate metastasis associated DNA sequences by PCR. With the LCM method, 5-7 $\mu$ m frozen sections are cut and freshly stained with H & E. These sections are examined and the areas of tumor cells are marked by Dr. Jones before LCM. A special transparent plastic cap, the bottom of which carries a transparent sterile thermoplastic film, is placed over the section. The slide is then placed on the microscope stage and cells that are clearly those of the tumor are selected. The diameter of the laser beam can be varied to cover one or more cells. This area is then pulsed briefly with a laser beam. This pulse heats the film and fuses the cells to it (Fig. 8). After many tumor cells have been captured in this way the cap is put on a 500 $\mu$ l PCR tube containing ATL lysis buffer (Qiagen Co.) and the DNA is extracted from the cells that are fused onto the outer surface of the cap.

#### **3. DNA extraction from microdissected cells.**

About 10,000 cells are microdissected by LCM from tumor tissue of sections. This microdissection takes about 25-30 minutes and DNA is extracted using a modified method of extraction from archival tissues (Achary et al. 2002; Mukherjee et al. 2002). Briefly, the microdissected cell pellet is incubated overnight (42°C) in Qiagen lysis buffer (Qiagen Co.) with

Proteinase-K at 55°C. Glycogen (carrier) is added to the cell lysate and DNA is extracted using phenol-chloroform-isomyl alcohol mixture followed by EtOH precipitation.

**4. Quantitative real-time PCR (Q-Rt-PCR):** Q-Rt-PCR analysis will be performed on the SmartCycler (Cepheid) using genomic DNA. An intercalating dye, SYBR Green is used in the real-time PCR, which binds to amplified products during the annealing and extension steps of each PCR cycle. The accumulation of amplified DNA is measured by the increase in fluorescence over time. The following reagents will be used for amplification in a Q-Rt-PCR: SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimized buffer components, from Applied Biosystems Inc., forward primer and reverse primer (final concentration: 50-900nM), cDNA template (1-100ng). PCR conditions will be: 95°C: 10min // 95°C: 15s, 55°C (changes with the melting temperature for each pair of primers used): 5s, 72°C: 10s (45 cycles) // 95°C: 5s, 65°C: 10s, Ramp 65-95°C at 0.1°C/s. The ratio between the amount of beta actin to the target gene corresponds to the gene dosage of both cDNA fragments (Hedrich et al. 2001). All detected gene expression variations will be confirmed at least twice. A ratio from 0.8 to 1.2 will be considered as normal, a heterozygous deletion is expected at a ratio between 0.4 and 0.6, and a homozygous loss at a ratio of 0 to 0.3. This method has recently been developed in the applicant's laboratory and was verified by using samples with known heterozygous and homozygous deletions.

#### 5. Fluorescent In Situ Hybridization (FISH):

FISH is a valuable technique used for localization and mapping of particular sequences of DNA, ranging from 1Kb to several Mb, on the chromosomes of cells and the nuclei of cells in the tissue sections (Cannizzaro and Shi, 1999). The sections of the ductal mammary carcinoma tissue samples (normal tissue/primary tumor/metastatic lymph nodes) will be processed and fixed on slides. The identification of the material will be done by PI in consultation with Dr. Jones and Dr. Russell. The selected samples will be processed for sectioning (microtomy) followed by brief staining with H and E by Dr. Russell. As mentioned elsewhere, attempts will also be made to prepare slides by touching the tumor tissue side on several places on the slide which will allow some cells to transfer from the block. For FISH studies, the slides containing sections or cells of ductal mammary carcinoma samples will be treated before hybridization with a paraffin pretreatment kit (Vysis Inc., Downers Grove, IL) according to the manufacturer's instructions. Similar treatment will be performed if the tissue is fixed in OCT. The sections will be air-dried and dehydrated by successive washes in 70%, 85%, and 100% ethanol and then incubated in 70% formamide and 2 x standard saline citrate (SSC) (1.75% sodium chloride/0.89% sodium citrate [pH 7]) for 5 minutes at 74 °C to denature the tumor DNA. Slides will be then incubated with a SpectrumOrange™-labeled BACs/MADS DNA probe and a SpectrumGreen™-labeled reference probe (PathVision™; Vysis Inc.) in hybridization buffer (2 g of dextran sulfate/10 mL of formamide in 2 mL 20 x SSC) overnight at 37 °C in a humidified chamber. After hybridization, the slides will be washed in 2 x SSC/0.3% Nonidet P-40, and the DNA will be counterstained with 0.2 µM 4,6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vectashield; Vector Laboratories, Inc., Burlingame CA). The slide will be analyzed by indirect immunofluorescence microscopy by use of a Zeiss Axiophot microscope equipped with Zeiss filtersets (Carl Zeiss, Göttingen, Germany) for DAPI, fluorescein isothiocyanate, and Texas Red (Carl Zeiss) under 100-fold magnification (Simon et al 2001).

#### 6. Retrofitting of BACs and transfection into MDA-MB-435 cells:

We identified 14 BACS that contain corresponding 14 MADS using gene bank search (Till to-date we could not find homology to MADS-IV in gene banks of NCBI and we do not have access to Celera database). We currently have obtained one BAC containing MADS-IX and the remaining will be purchased from Research Genetics (Huntsville, AL). The candidate BACs will be retrofitted with a selectable marker *neo* and then introduced individually into metastatic human mammary MDA-MB-435 tumor cells following the procedure described by Mejia and Monaco (1997).

Briefly, BAC DNA is digested with Not I and used for ligation with dephosphorylated, Not I linearized retrofitting vector pJMOx166. This vector carries the *cat* gene for selecting chloramphenicol resistance in *E.coli* cells and *neo* gene for selection of mammalian cells in G418. Ligated DNA was dialyzed and transformed into *E.coli* DH10B cells. The transformation mix was plated on LB medium containing 30ug/ml kanamycin and 20ug/ml chloramphenicol. The resulting transformants will be analyzed by PCR (with MADS primers) to confirm the presence of the MADS. Selected clones were then fingerprinted using HindIII digestion to confirm the integrity of the human insert in retrofitted BACs.



BAC-DNA is isolated from cultures of retrofitted BACs by alkaline lysis and Qiagen column purified. Purified BAC DNA was introduced into immortal human mammary carcinoma (MDA-MB-435) cells using lipofection and electroporation methods. Both methods will be used parallel to recover large number of clones for further analysis. For electroporation, 5ug of BAC DNA is mixed with  $1 \times 10^7$  recipient cells in a 0.4cm gap cuvette and pulsed at 300 volts with 500 or 960 uF capacitance. For lipofection, 10ug of BAC DNA is mixed with 300ul of lipotaxi reagent (Stratagene) and layered on top of a monolayer of  $5 \times 10^5$  recipient cells. After 48 hours of growth in non-selective medium, recipient cells are fed with medium (DF12) supplemented with 10% FBS and containing 500ug/ml of G418. G418 resistant colonies, which appeared in the ensuing 3 week period, were either isolated individually in separate plates or followed in the parent plates. Colonies were observed for morphology and cell growth characteristics and photographed at regular interval.

## 7. Evaluation of metastatic potential of BACS/MADS:

**In vitro assay:** The cell invasion assay is based on the principle of Boyden Chamber (Albini et al., 1987; Yang et al., 2001). Biocoat Matrigel invasion chambers are purchased from Becton Dickinson, and the protocol is provided by the manufacturer. Briefly, cells are plated in the top chamber ( $1.5 \times 10^4$  cells/chamber). An 8- $\mu$ m pore size Matrigel-coated polycarbonate filter separated the top and bottom chambers. The bottom chamber contained 5% FBS as a chemoattractant. After 24-h incubation, the noninvasive cells are removed with a cotton swab. The cells that had migrated through the membrane and stuck to the lower surface of the membrane are fixed with methanol and stained with hematoxylin. For quantification, cells are counted under a microscope in five predetermined fields at x200.

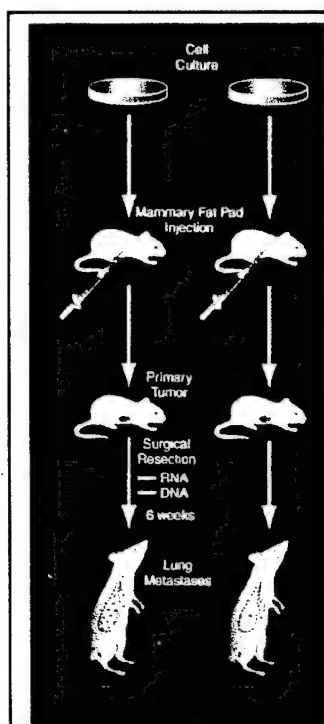


Fig. 12. *In Vivo* Spontaneous Metastasis Model: The left panel represents the mouse injected with control cells (vector only) and right panel with a retrofitted BAC/MADS which has the potential to inhibit or reduce metastasis.

***In Vivo* Metastasis Assay:** MFP model (Fig. 12): Human mammary carcinoma cells, MDA-MB-435 transfected with BAC/MADS are injected ( $@1 \times 10^6$ ) into axillary MFPs of anesthetized 5- to 6-week-old female SCID mice. Tumor sizes were monitored a week after inoculation of tumor cells. When the mean tumor diameter reaches 1.0 cm, tumors are surgically removed. Four weeks later, one set of mice (5) are sacrificed and lymph nodes are examined. After 8 weeks second set of mice (5) are sacrificed and visible lung metastases counted. Lymph nodes and lungs are removed; half snap frozen and half fixed in Bouin's solution for quantification of surface metastases as described (Welch, 1997). Animals are maintained under the guidelines of the NIH and approved protocols of IACU Committee of AECOM (Copy of the approval letter enclosed)

### Tumor histology and quantitative assessment of the efficiency of lung micrometastasis.

The primary tumors, lymph nodes and lungs from each mouse are used for histological analysis. Samples are fixed in 10% neutral formaline buffer, embedded in paraffin, and sectioned at 5  $\mu$ m. Sections are stained by H&E and reviewed and number of tumor cells/lung micro-metastasis scored by PI and Dr. Russell. For each lymph node and lung sample, all micro-metastases are counted under light microscope at 10X magnification and the total lung area is measured by UMAX PowerLook III color scanner (UMAX Technologies, Inc., Dallas, TX) and Adobe Photoshop 5.5 software. Briefly, after scanning the lymph node and lung sections, the cross sectional area in pixels were measured using PhotoShop. The number of pixels are read from the window of Luminosity. The actual lung tissue area is calculated with the aid of the pixel-metric conversion in the formula: {Lung tissue area ( $\text{mm}^2$ )=numbers of pixelX0.001789284}. The efficiency of lung metastasis is expressed in metastases number per  $\text{mm}^2$  of lung area.

### **Statistical analyses:**

Cell invasion: Cell invasiveness is measured as the number of invaded cells per 5 fields. The comparison is made using ANOVA methods with a single contrast of 435

control and 435-BAC/MADS transfected cells.

**Metastasis:** The number of lymph node or lung metastasis in the BAC/MADS transfectant is compared with control using Mann-Whitney rank-sum test (Zar, 1996). Means and SEs, as well as the ranges for those mice with metastasis, are reported for each. Each test is considered significant if the  $P$  is  $<0.05$ . All of the analysis will be performed using STATISTICA software (Statsoft, Inc., Tulsa, OK).

## Methods (II):

(Methods that were exclusively used in the original grant application to generate preliminary data presented in this revised application and will not be used in this revised proposal)

### 1. Representational Difference Analysis (RDA) method:

The RDA method combines three elements: representation, subtractive enrichment and kinetic enrichment. Two to three rounds of hybridization/selection are used to achieve full purification of the difference products. Only important steps in the RDA method are presented in the diagram (Fig. 13; see Lisitsyn and Wigler, 1995 and our results in Fig. 2 a, b and c). Using 11 RDA experiments we isolated 15 candidate MADS. We do not have intentions at present to perform additional RDA experiments.

Fig. 12. Schematic representation of RDA method showing representation step, subtractive hybridization (1-3 rounds) followed by enrichment of differential target gene sequences.

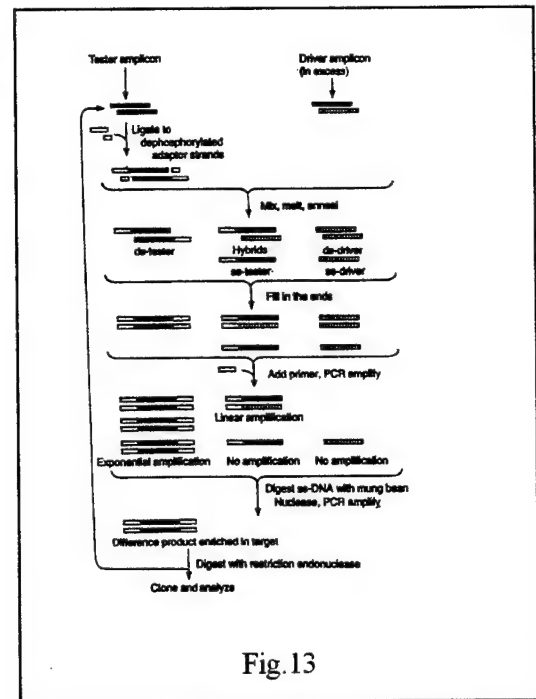


Fig. 13

### 2. Isolation of cells from biopsy samples by single cell microdissection (SCM).

The RDA procedure requires about 95% purity of driver and tester DNA. For this reason previous workers used cell lines derived from fresh tissues (Lisitsyn et al., 1995; Li et al., 1997) or xenografts (Schutte et al., 1995). Both of these methods however, are not suitable for archival tissues. We therefore used SCM (Mukherjee et al. 2002) to obtain pure population cells to perform RDA experiments. SCM was performed on hematoxylin and eosin stained tissue sections of primary tumor and positive lymph nodes from the ductal breast cancer patient samples (Fig. 1). Currently we are using only LCM.

### 3. Physical mapping of MADS by RH mapping:

**Radiation Hybrid (RH) Mapping:** RH mapping is used to localize the MADS on human chromosomes using high resolution Gene Bridge 4 radiation hybrid panels (Research Genetics, Inc.). RH mapping is a somatic cell hybrid technique that was developed to construct high-resolution contiguous maps of mammalian chromosomes. The distance provided by this method is directly proportional to the physical distance. RH mapping uses a statistical program (RHMAP) that will provide the best map along with a measure of the relative likelihood of one order versus another of sequences by Radiation Hybrid mapping (<http://www.sph.umich.edu/group/statgen/software>). The RH panel of 93 radiation hybrid clones represent the whole human genome. This panel is a subset of 199 clone panel developed by Walter et al (1994). A human cell line was exposed to 3000rad of X-rays and then fused with thymidine-deficient hamster recipient cell, creating a panel of hybrids with around 1000 kb resolution.

Based on the sequences of differential products isolated from RDA experiments, primers are designed for each group of candidate gene sequences. For RH mapping PCR reactions are run using 94 DNA templates and primers specific to the sequence being mapped along with a positive control (human genomic DNA) and two negative controls (Chinese hamster genomic DNA and no template). The results are arranged as suggested by Research Genetics Inc. (positive bands denoted as 1; negatives denoted as 0 and doubtful ones denoted as 2). The databases at Whitehead Institute/ MIT (<http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper>) were used to obtain the chromosome localization of these sequences.

**4. Screening of multiple human tissue Northern blots:**

To determine if a MADS is an expressed sequence or not and if expressed, is it tissue specific (expressed only in breast tissue) and tumor-specific, Human multiple tissue RNA Master Blots<sup>TM</sup> (CLONTECH Laboratories, Inc.) could be used. These are positively charged nylon membranes to which poly A+RNA from different human tissues and developmental stages have been immobilized in separate dots, along with several controls. It provides a fast, easy method for obtaining accurate information about the expression of a cloned gene of interest across a wide range of tissues and developmental stages. A MADS can be used as a  $\alpha$ 32p-dCTP probe to hybridize with the RNA Master Blot, following standard Northern blotting protocol. Results will reveal if a MADS is tissue specific or expresses in all the tissues. In either case it will indicate if it is a part of a gene that transcribes. Ubiquitin gene is used as an internal control.

**5. Isolation of partial and full-lengths of MADS:**

Most of the RDA recovered MADS range in sizes from 100-300 bp. In order to use MADS as FISH probes to screen patient tissue sections, one needs either full-lengths or at least partial sequences (more than 2Kb). To accomplish this, inverse-PCR (I-PCR) and human mammary cDNA library screening are proposed.

**i. Inverse-PCR:** I-PCR is generally used to analyze DNA sequences either upstream or downstream from a known sequence (Li et al., 1999). Normal human genomic DNA is digested and the DNA fragments are allowed to self ligate using T4 DNA ligase to form self-circularized sequences. This circular DNA is used as templates in PCR with newly designed forward (inverse X-1), and reverse primers (inverse X-2) using the Expand Long Template PCR System (Roche). The required PCR program is as following: At 94 °C for 2 min, followed by 10 cycles of 94 °C for 10 s, 52 °C for 30 s, 68 °C for 5 min, then 20 cycles of 94 °C for 10 s, 52 °C for 30 s, 68 °C for 5 min with a 20s auto extension, and a final extension step at 68 °C for 10 min. The PCR products are run on an agarose gel and DNA is extracted from the top band (2-10Kb) and cloned into TOPO TA system (Invitrogen). Sequences more than 1 Kb size could be used as FISH probes.

**ii. Human mammary cDNA library screening:**

To isolate full-length cDNA of a MADS, a human breast adenocarcinoma library such as cDNA  $\lambda$ gt11 library (CLONTECH Labs, Inc., Cat# HL1099b) could be screened. Briefly, different dilutions of the lambda library is plated on LB plates and plaques are transferred onto membranes and screened with MADS as  $\alpha$ 32p-dCTP probe. After three rounds of screening, positive plaques are isolated and pure stocks are prepared. The clones are characterized and sequenced to verify if they contain the original MADS. These cDNAs can be used as FISH probes and also for functional studies in breast metastatic cell line/SCID xenograft mouse models.

**iii. Isolation of full-length cDNA of MADS by BAC library screening:**

In order to use the MADS isolated in our RDA experiments as FISH probes, we propose to screen high density filters containing bacterial artificial chromosomes (BAC) and obtain the clone that consists of the MADS. These membrane filters are available from BACPAC Resources of Children's Hospital Oakland Research Institute, Ca. Following the procedure of the manufacturer, filters are processed for hybridization and MADS labeled as 32p dCTP probe by random prime labeling. Hybridize the membrane filters overnight at 65C and expose to film after washing. The positive clones are verified by PCR and sequencing if they contain the MADS. Then the BAC could be used as probe for FISH studies.

**e. Human subjects:**

A total of 80 archival ductal breast cancer tissues will be analyzed in this investigation. These samples consist of primary tumor, and positive lymph nodes (40 cases) and primary tumors without lymph node (40 cases) samples from individual patients. Computerized case histories of all the cases including data on the patients' pathological parameters, will be available to this investigation. However, this information is made available to the investigators in such a manner that subjects of this study cannot be identified directly or through linking identifiers. These patient samples are selected without any bias for race or age. Most importantly, the P.I. and the laboratory staff will be blinded with respect to the clinical response of the patients during the molecular studies. Successful completion of the proposed studies will lead to

prospective studies in future grant projects that will assess genetic and environmental factors that relate to race, including attempts to understand why breast cancer has higher frequency and severity in women of certain groups.

### **Precautions during the study:**

During this investigation the P.I. and other associates in this project have to handle radioactive isotopes and human breast carcinoma tissue samples. The P.I., and all co-investigators and the Research Associate of this proposal have been qualified through the Radiation Safety and Handling of Infectious Agents training courses and have been vaccinated against hepatitis B. Any new personnel that may join this project will participate in these safety-training courses and all will follow the necessary precautions as prescribed in these courses.

### **Gender and Minority inclusion:**

This study includes tissues of breast cancer patients without any bias for their race, color, age or ethnicity.

### **f. Vertebrate animals:**

**Description of proposed animal use.** The human mammary adenocarcinoma cell line (MDA-MB-435) transfected with BACs/MADS will be used to generate primary tumors and metastases in female immunocompromised mice (Rag-2 -/- or scid/scid). We estimate that up to 30 mice per year might be used. For each transfected cell line to be tested, up to 10 mice will be used to generate primary tumors in the mammary fat pad. This cell line is widely utilized and have shown no effects or risk to personnel. We estimate that up to 2 BACs/MADS will be tested per year by transfecting this cell line [(2 test BACs/MADS + BAC control) X 10 mice per group] = 30 mice per year.

For injection into the mammary fat pad, mice will be gently restrained and then 0.1 - 0.3 ml of MEM containing 500,000 cells will be injected in the mammary fat pad. The animals will then be housed in the animal facility for 6 -10 weeks for generation of primary tumors and metastases. For generation of lung metastases, the primary tumor will be removed after the tumor reaches 1 cm in diameter. In this case, the animal will be anesthetized with isoflurane, the primary tumor removed surgically, and then the wound closed with sterile wound clips. The animal will then be allowed to continue for an additional 4 weeks to allow lymph node and lung metastases to grow, or until the animal displays clear signs of metastases, such as dyspnea at which time the animal will be sacrificed by carbon dioxide overdose and tissues (lymph nodes and lungs) collected for analysis. To monitor the stress on the animal during tumor or metastasis growth, the animals will be checked by the Research Associate from our lab every day.

1. **Justification for animal use.** Metastasis is a complex process that involves detachment from adjacent cells and extracellular matrix, cell motility, translocation, reattachment, growth and angiogenesis. The full process can only be examined in vivo. Therefore animals must be used. Because we are interested in the functional aspect of human metastasis associated genes in human breast cancer we are using human mammary adenocarcinoma cell xenografts in mice. Mice with the severe combined immunodeficiency phenotype (scid/scid) readily accept human tissue grafts. These mice are a well established and tractable model for tumorigenicity and metastasis assays.
2. **Veterinary care.** Four full-time veterinarians participate in our institution's program of care and use. Veterinary care includes a program for prevention of disease, daily observation and surveillance for assessment of animal health; appropriate methods of disease control, diagnosis, and treatment; guidance of animal users in appropriate methods of handling, restraint, anesthesia, analgesia, and euthanasia; and monitoring of surgical programs and post-surgical care.
3. **Procedures for minimizing discomfort, distress, pain, and injury.** For injection into the mammary fat pad in spontaneous metastasis assays, there is no requirement for anesthesia, and the injection itself does not cause more than transitory discomfort. Mice will be monitored frequently and any mouse experiencing respiratory difficulty or severe weight loss will be euthanized.
4. **Euthanasia method.** Euthanasia of animals will utilize carbon dioxide overdose. Once mice are unconscious and in respiratory arrest major organs such as lungs and liver will be removed, ensuring death. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Association.



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**h. Consortium:** Not applicable

**i. Consultants/ Collaborators:** (letters enclosed)

1. Dr. Joan G. Jones, AECOM/MMC.
2. Dr. Abdissa Negassa, AECOM.
3. Dr. Robert Russell, AECOM
4. Dr. Abdel H.A.A.Wahab, Cairo University
5. Dr. R.S. Athwal, Temple University.

MONTEFIORE MEDICAL CENTER

Jack D. Weiler Hospital  
of the Albert Einstein  
College of Medicine

A Division of Montefiore Medical Center

1825 Eastchester Road  
Bronx, New York 10461-2373

718

718-904-2947

June 24th, 2002

Dr. P. M. Achary  
Assistant Professor  
Department of Radiation Oncology  
Albert Einstein College of Medicine  
Bronx, NY.

MONTEFIORE



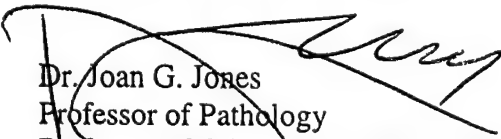
Dear Dr. Achary,

I am happy to serve as the collaborating pathologist for your project on the characterization of metastasis associated DNA sequences in ductal mammary carcinoma. I understand that as a collaborator, I will receive 5% salary support. As you know, I have a longstanding interest in the progression of breast cancer and, in particular, mechanisms of metastasis. Furthermore, as a senior level, actively practicing Surgical Pathologist, I have extensive experience in the evaluation of human breast cancer pathology.

I will reconfirm the histopathology of the tissue specimens obtained from the CHTN and other sources and also will provide you with additional samples from archival tissues maintained by the Department of Pathology of the Albert Einstein College of Medicine and Montefiore Medical Center. Together we can reconfirm previously reported observations. I will also personally supervise you and your research personnel in the identification and confirmation of pure tumor cell populations obtained by laser capture microdissection (LCM).

I have read the critique of your NIH R-21 grant application and understand the concerns of the reviewers. As discussed with you and Dr. Negassa, the collaborating Biostatistician, cases will be analyzed for appropriate clinically relevant parameters including tumor size, number of positive lymph nodes, hormone receptor and Her-2neu status.

I look forward to an exciting and productive collaboration.

  
Dr. Joan G. Jones  
Professor of Pathology  
Professor of Clinical OB/GYN & Women's Health  
Albert Einstein College of Medicine  
Director, Surgical Pathology  
Einstein Division, Montefiore Medical Center

Albert Einstein College of Medicine  
of Yeshiva University  
Department of Epidemiology & Social Medicine

Jack and Pearl Resnick Campus • 1300 Morris Park Avenue • Bronx, New York 10461 USA

Abdissa Negassa, Ph.D.  
Assistant Professor

Phone: (718) 430 - 3575  
Fax: (718) 430 - 8780  
Email: anegassa@aecom.yu.edu

June 26, 2002

Dr. Mohan P. Achary  
Assistant Professor and Cancer Biologist  
Department of Radiation Oncology  
Albert Einstein College of Medicine  
Bronx, NY

Dear Dr. Achary,

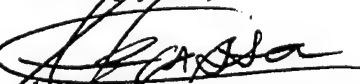
I am happy to serve as a biostatistical consultant with 5% of effort in your project on "Markers of Metastasis in Ductal Mammary Carcinoma" which is being re-submitted to NIH.

My experience as study biostatistician with BBD and P53 mutation studies here at Einstein and my familiarity with multivariable analysis will enable me to provide statistical input into your study both at the design as well as analysis stages. I will be available to help you with the statistical aspects of your study. I believe that your study has a great translational potential for better assessment of prognosis as well as designing better therapeutic strategies.

I went over the reviewers' comments of your NIH R-21 grant application and understood the concerns of the reviewers. Based on my discussion with you and Dr. Jones, the collaborating Pathologist, now we have clearly specified the major clinical parameters that are going to be included in the multivariable analysis.

I look forward to a productive and fruitful collaboration with your team.

Sincerely yours,



Abdissa Negassa, Ph. D.

Assistant Professor

Department of Epidemiology and Social Medicine  
Albert Einstein College of Medicine

ALBERT EINSTEIN COLLEGE OF MEDICINE  
OF YESHIVA UNIVERSITY  
JACK AND PEARL RESNICK CAMPUS  
1300 MORRIS PARK AVENUE, BRONX, NEW YORK, 10461.  
Histotechnology and Comparative Pathology Facility  
Forchheimer Rm #734 / Office Phone - (718) 430-3209

June 19th, 2002

Dr. Mohan P. Achary  
Department of Radiation Oncology  
Albert Einstein College of Medicine  
1300 Morris Park Avenue  
Bronx, NY 10461

Dear Dr. Achary:

I am delighted for the opportunity to collaborate with you on your studies to characterize and to evaluate the candidate metastasis associated DNA sequences (MADS) in breast carcinoma.

This support for your studies is provided under the services offered by the core Histotechnology and Comparative Pathology Facility at Albert Einstein College of Medicine. This institutional core facility is affiliated with the Comprehensive Cancer Center and provides support for pathology evaluations and interpretation, particularly in experimental animal model studies. I am a board certified veterinary pathologist with extensive experience in animal model studies and particularly models for studies of cancer mechanisms. I also have experience with examination of human tumors. I will help you with your experiments involving SCID mice to evaluate the metastatic potential of the cells transfected with BACs containing candidate metastasis associated genes.

The core facility will provide full custom histological laboratory services for the histological preparations required for your studies including sectioning and staining human mammary and metastasis tumors for laser capture microdissection or for FISH studies and similar studies of tumors from the mouse studies including processing.

I am experienced in identifying tumor cells from lymph nodes of mice. Therefore we will be able to differentiate lymph node metastasis from lung metastasis in your SCID mice model. My experience with pathology of human tumors from my previous and current interactions with MD pathologists, together with my experience with experimental animal tumor pathology provide me with the experience and resources to support your

studies. I will be happy to provide you additional pathology consultation services throughout the course of your studies.

Sincerely,

A handwritten signature in black ink, appearing to be 'R. Russell', written over the printed name.

Dr. Robert G. Russell. BVSc., Ph.D., ACVP  
Scientific Director, Histotechnology and Comparative Pathology Facility  
Associate Professor, Department of Pathology





Dated: 6-25-02

Mohan P. Achary Ph.D.  
Albert Einstein College of Medicine  
1300 Morris Park Avenue  
Bronx, NY 10461

Dear Dr. Achary:

I am delighted to collaborate with you on your NIH grant to identify and characterize metastasis associated DNA sequences (MADS) in ductal breast carcinomas. I am happy to note that you have isolated couple of MADS and proposing to screen a larger population of breast carcinoma patient tissue samples to determine if they could be used as prognostic markers for the lymph node negative breast carcinomas.

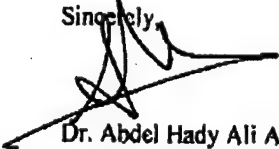
For the past several years our Pathology department has been collecting primary breast tumors and matched paraffin embedded positive and negative lymph node material. We have more than 100 such cases in my department and I will be happy to provide you with DNA or tissue blocks of those cases as per your requirement. As suggested by your Pathologist I will also provide you with the clinical information such as tumor size, lymph node status, ER/PR and Her2/neu of each of the tumor. We are now collecting fresh frozen samples and you can also have access to them in near future for the cDNA microarray studies.

I learnt that you have already screened one of your MADS by PCR on 10 DNA samples I sent to you and found that it is lost in 2 cases. I will mail you another set of 10 cases as soon as possible. I have also plans to visit your Institute between July and August this year. At that time I will try to bring as many tumor samples as possible for the present collaborative investigation.

As per our recent discussion please include in your proposal the expenses that are expected in my department at Cairo University, towards cutting the tumor sections, processing, preparation of slides ready for LCM and required chemicals, reagents etc.

Best of luck with your grant application.

Sincerely,

  
Dr. Abdel Hady Ali Abdel Wahab  
Cancer Biology Department  
National Cancer Institute  
Foam El-Khalig, Kasr Al-Aini  
Cairo University, Cairo, Egypt.



TEMPLE UNIVERSITY  
A Commonwealth University

School of Medicine  
Fels Institute for Cancer  
Research and Molecular Biology

Medical Research Building  
3420 N. Broad Street  
Philadelphia, Pennsylvania 19140  
(215) 707-4300  
Fax: (215) 707-4318

June 22nd, 2002

Mohan Achary, Ph.D.  
Albert Einstein College of Medicine  
1300 Morris Park Avenue  
Bronx, NY 10461

Dear Dr. Achary:

I am happy to collaborate on your NIH grant on the identification and characterization of metastasis associated DNA sequences (MADS) in ductal breast carcinomas.

I understand that you have isolated several MADS and future work is to evaluate these DNA sequences for their role in metastasis. An easier experimental system would be to study the suppression of cell growth and metastasis potential, following the introduction of BAC clones, corresponding to each MAD clone, into MDA-MB-435 cells. We routinely perform these types of experiments in our effort towards the cloning of cell senescence genes. We will be very happy to provide any type of experimental help that you might need in accomplishing the objectives of your project.

Best of luck.

Sincerely Yours,

Raghubir S. Athwal, Ph. D.  
Professor

Department of Pathology And Laboratory Medicine  
Fels Institute For Cancer Research and Mol. Biology  
Temple University School of Medicine  
AHB Room 202  
3307 North Broad Street  
Philadelphia, PA19140

## CHECKLIST

## TYPE OF APPLICATION (Check all that apply.)

☐ NEW application. (This application is being submitted to the PHS for the first time.)☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. \_\_\_\_\_☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. \_\_\_\_\_☐ SBIR Fast Track☐ STTR Fast Track☒ REVISION of application number: 1R21 CA097208-01

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number: \_\_\_\_\_

(This application is to extend a funded grant beyond its current project period.)

## INVENTIONS AND PATENTS

(Competing continuation appl. and Phase II only)

☐ No☐ Previously reported☐ SUPPLEMENT to grant number: \_\_\_\_\_

(This application is for additional funds to supplement a currently funded grant.)

☐ Yes. If "Yes,"☐ Not previously reported☐ CHANGE of principal investigator/program director.

Name of former principal investigator/program director: \_\_\_\_\_

☐ FOREIGN application or significant foreign component.

## 1. PROGRAM INCOME (See instructions.)

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

## 2. ASSURANCES/CERTIFICATIONS (See instructions.)

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells•  
 •Research on Transplantation of Human Fetal Tissue •Women and  
 Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals•

•Debarment and Suspension; •Drug-Free Workplace (applicable to new  
 [Type 1] or revised [Type 1] applications only); •Lobbying; •Non-  
 Delinquency on Federal Debt; •Research Misconduct; •Civil Rights  
 (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641  
 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age  
 Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and  
 Human Gene Transfer Research; •Financial Conflict of Interest (except  
 Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution  
 Participation.

## 3. FACILITIES AND ADMINISTRATIVE COSTS (F&amp;A)/INDIRECT COSTS. See specific instructions.

☒ DHHS Agreement dated: 1/23/2002☐ No Facilities And Administrative Costs Requested.☐ DHHS Agreement being negotiated with \_\_\_\_\_

Regional Office.

☐ No DHHS Agreement, but rate established with \_\_\_\_\_

Date \_\_\_\_\_

CALCULATION\* (The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)

a. Initial budget period:	Amount of base \$	<u>98,000</u>	x Rate applied	<u>67</u>	% = F&A costs	\$	<u>65,660</u>
b. 02 year	Amount of base \$	<u>100,000</u>	x Rate applied	<u>67</u>	% = F&A costs	\$	<u>67,000</u>
c. 03 year	Amount of base \$	_____	x Rate applied	_____	% = F&A costs	\$	_____
d. 04 year	Amount of base \$	_____	x Rate applied	_____	% = F&A costs	\$	_____
e. 05 year	Amount of base \$	_____	x Rate applied	_____	% = F&A costs	\$	_____
TOTAL F&A Costs \$							<u>132660</u>

\*Check appropriate box(es):

☐ Salary and wages base☐ Modified total direct cost base☐ Other base (Explain)☐ Off-site, other special rate, or more than one rate involved (Explain)

Explanation (Attach separate sheet, if necessary.):

4. SMOKE-FREE WORKPLACE ☒ Yes ☐ No (The response to this question has no impact on the review or funding of this application.)

ALBERT EINSTEIN COLLEGE OF MEDICINE  
OF YESHIVA UNIVERSITY  
JACK & PEARL RESNICK CAMPUS  
Belfer Educational Center for Health Sciences, Room 1002  
1300 Morris Park Avenue, Bronx, NY 10461

Committee On Clinical Investigations  
(Institutional Review Board)

Phone: (718) 430-2237  
Facsimile Number: (718) 430-8817

November 14, 2001

Mohan P. Achary, Ph.D.  
Department of Radiation Oncology

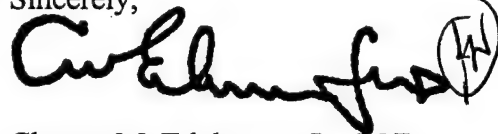
RE: CCI No.96-119 FWA: 00000140 MMC No.95 11 443  
TITLE: ISOLATION OF NOVEL GENETIC LESIONS IN CANCER PATIENTS BY  
REPRESENTATIONAL DIFFERENCE ANALYSIS OF ARCHIVAL TISSUES aka  
1. MOLECULAR MARKERS OF METASTASIS IN DUCTAL MAMMARY  
CARCINOMA 2. MARKERS FOR SENSITIVITY OF CERVICAL CANCERS TO  
THERAPIES

Dear Dr. Achary:

This is to inform you that the Committee on Clinical Investigations (CCI) has reviewed and recertified the above referenced human research project for the period from November 17, 2001 to November 16, 2002. The study was included in the CCI agenda for the meeting of November 14, 2001.

Should you have any questions regarding the above, please contact Ms. Patricia Hopkins at (718) 430-2237.

Sincerely,



Chester M. Edelman, Jr., M.D.  
Chairman  
Committee on Clinical Investigations

Animal Institute Committee  
Albert Einstein College of Medicine  
1300 Morris Park Avenue  
Bronx, New York 10472

06/13/2001

Dr. Mohan Achary  
Radiation Oncology

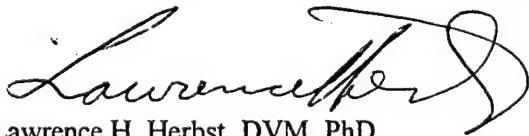
Project Title: An In Vivo Model to Study Metastasis in Breast Cancer

Animal Institute Committee Protocol # 000611

Dear Dr. Achary:

The animal use protocol for the above project was reviewed and approved by the Animal Institute Committee of Albert Einstein College of Medicine, our institution's animal care and use committee, on 07/19/2000. Any significant changes in personnel, the procedures performed on animals, additional numbers of animals, or use of other species must be reviewed and approved by the committee.

Animal Institute Committee approval is for a period of three (3) years from the approval date. Protocols involving USDA species must be reviewed and reapproved annually. For other protocols, approval is extended on an annual basis for up to three (3) years following administrative review of a brief update form. Please refer to the above AIC project number when modifying or renewing this protocol and when ordering animals.



Lawrence H. Herbst, DVM, PhD  
Animal Welfare Officer  
Albert Einstein College of Medicine  
Telephone (718) 430-8553 FAX (718) 430-8556  
E-mail: herbst@aecom.yu.edu

**2 ABSTRACTS presented in AACR meetings**

**3 Published papers**

✓ **2 Submitted manuscripts** (Not attached with this copy)



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APRIL 10-14, 1999 PHILADELPHIA, PA

Volume 40 March 1999

In cooperation with



discriminate score for each cell represented the severity of nuclear atypia. The distribution of this score over cells from a given lesion was analyzed in terms of lesion heterogeneity and frequency of high-grade cells. The consistency of results using these preliminary methodologies was encouraging. Correlative analysis of morphometric and immunohistochemical markers is planned for a much larger independent series. In chemoprevention studies, nuclear morphometry may serve as a surrogate endpoint biomarker, allowing for characterization of the extent and severity of nuclear atypia and for evaluation of treatment response in terms of a reduction in nuclear grade.

**#2845 Search for novel molecular markers to identify patients with breast cancer at high risk for developing metastases using representational difference analysis.** Achary, P.M., Mukherjee, B., Khaimov, R., Fan, Z., Mahadevia, P.S., and Vikram, B. Department of Radiation Oncology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY 10461.

Using single cell microdissection, we isolated normal cells and cancer cells from a metastatic lymph node from a woman with breast cancer. Representational difference analysis (RDA) was performed to isolate genomic DNA sequences that had undergone alteration during progression from normal to metastatic phenotype. RDA has yielded 11 differential sequences. Dot/Southern blot analyses have confirmed that these sequences were present in normal but lost from the metastatic cell DNA. The preliminary characterization of one of the novel sequences, M-41, has been undertaken in another four patients with invasive ductal primary breast cancers with lymph node metastases. From each patient's specimens, ~1000 cells each were microdissected from the normal tissue, the primary tumor and the metastasis. Genomic DNA was isolated from each of these 12 samples, and amplified by a method recently standardized in our laboratory. In each of the 4 patients, we have observed strong hybridization signals from the normal and primary tumor DNA, and only faint signals from the metastatic DNA, suggesting a potential metastasis-specific genetic marker. In addition to repeating these studies, we are screening few more patients' specimens.

**#2846 Predicting recurrent ductal carcinoma in situ (DCIS) of the breast: Nuclear image analysis feature.** Hoque, A., Lippman, S.M., Atkinson, N., Boiko, I., Sneige, N., Sahin, A., Sabichi, A.L., Weber, D., Lagios, M., Schwartz, R., Colburn, W., Dhingra, K., Kelloff, G., Silverstein, M., Boone, C., and Hittelman, W.N. The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

This retrospective multi-center case control study evaluated nuclear morphologic features (MFs) measured by image analysis (IA) of biopsy specimens of 34 DCIS lesions associated with subsequent recurrence (cases) and 73 DCIS lesions without recurrence (controls). IA was performed on Feulgen stained slides using the CytoSavant instrument. Data distribution of 46 morphologic nuclear shape features (NSFs) was categorized into four groups using cut points corresponding to  $\leq 25^{\text{th}}$  (referent category),  $> 25^{\text{th}}$  and  $\leq 50^{\text{th}}$ ,  $> 50^{\text{th}}$  and  $\leq 75^{\text{th}}$ , and  $> 75^{\text{th}}$  percentile. After adjustment for age at diagnosis, length of follow-up, radiotherapy, nuclear grade and necrosis, an increasing dose response relationship was observed between recurrence of DCIS and higher value of the following NSFs: eccentricity, var.radius, inertia.shape, freq.low.fft, freq.high.fft, harmon.01.fft and harmon.02.fft. For example, adjusted odds ratios (ORs), (confidence intervals, CIs) for each category of var.radius compared to referent group were 1.33 (95% CI 0.33-5.37), 3.25 (95% CI 0.75-13.96) and 5.23 (95% CI 1.02-26.86), respectively. ORs for the  $> 75^{\text{th}}$  percentile category of eccentricity, inertia.shape, freq.low.fft, freq.high.fft, harmon.01.fft and harmon.02.fft were 4.36 (95% CI 0.91-20.72), 3.03 (95% CI 0.84-10.90), 3.54 (95% CI 0.96-13.09), 3.20 (95% CI 0.92-11.16), 3.97 (95% CI 1.01-15.55) and 3.6 (95% CI 0.98-13.15), respectively. These results indicate that NSFs might provide additional predictive information for the risk of recurrence. (NCI N01-CN-65004)

**#2847 Image analysis as predictive factor for recurrence of ductal carcinoma in situ.** Boiko, I.V., Hoque, A., Sneige, N., Atkinson, N., Sahin, A., Sabichi, A.L., Weber, D., Lagios, M., Schwartz, R., Colburn, W., Dhingra, K., Kelloff, G., Silverstein, M., Boone, C., Lippman, S.M., and Hittelman, W.N. The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

As part of a large retrospective case control study of recurrent DCIS, a subset of 10 recurrent and 10 non-recurrent DCIS with negative margins were evaluated by image analysis to determine the fractal texture features in DCIS lesions and adjacent normal-appearing tissue. The case and control specimens were matched on nuclear grade, age at diagnosis, radiotherapy and length of follow up. An experienced pathologist mapped each slide for DCIS regions and normal-appearing regions 2mm away from DCIS lesions. Feulgen stained samples were then analyzed by image analysis using the CytoSavant instrument. Texture features from 200 nuclei from each region of interest (total 400 nuclei per slide) and 100 lymphocytes as internal control were determined. Fractal 2 area, a feature describing the area of the three-dimensional surface of the object's optical density was found to be the best discriminant factor between recurrent and non-recurrent DCIS nuclei. Fractal 2 area was increased in cases with recurrence compared to that of non-recurrent DCIS. The value of this feature increased in DCIS regions (969  $\pm$  155 vs 821  $\pm$  101, respectively, P-value 0.13). This trend was even stronger in the nuclei of adjacent normal-appearing ducts in the cases as compared to the controls (629  $\pm$  31 vs 489  $\pm$  23, P-value 0.01). These results

suggest that chromatin texture features in normal-appearing ducts in the field of DCIS lesions might provide prognostic information of the risk of recurrence. (Supported in part by NCI N01-CN-65004).

**#2848 Psychological outcomes of Jewish women in a high-risk cancer program.** Daly, M., Harrop-Stein, C., Malick, J., Godwin, A., Dangel, J., Ross, E. and Siemers, S. Fox Chase Cancer Center, Philadelphia, PA 19111.

Among the factors associated with breast cancer, none, other than gender and age, alters the magnitude of risk more than a family history. Three mutations, 185delAG, 5382insC and 6174delT, predisposing to hereditary breast and ovarian cancer have been detected in Jews of Eastern European descent. Until recently, little research has been done to study the psychological sequelae of cancer genetic test results. The Family Risk Assessment Program surveyed Jewish women about their perceptions of genetic testing and the psychological factors associated with potential gene carrier status. Sixty women completed the Beck Depression Inventory (BDI) the Revised Impact of Events Scale (RIES) and Cancer Risk and Worries at baseline, post counseling, and 1 and 4 months post disclosure. The sample was stratified into two groups, those with a family history of cancer (A) and those with out a family history (B). Overall, our sample scored at the normative population means for the BDI and RIES. There were no significant differences between the two groups for the BDI. Total RIES scores at baseline show that those in group A have a higher level of anxiety related to their risk of cancer than those in group B. However, by four months post-disclosure both groups of women have low mean RIES scores. Women were asked to rate their chances of developing breast/ovarian cancer on a scale of 0%-100%. Women in group A rated themselves significantly higher (60% risk) than group B (40% risk;  $p = .0016$ ). There was significant improvement in overall risk perception from baseline to four months ( $p = .0011$  and  $.035$  respectively). As one would expect, those with a family history feel more vulnerable to breast and ovarian cancer. These results also suggest that the Jewish women seeking genetic testing are well adjusted and cope well with test results.

**#2849 Evaluation of biomarker modulation by fenretinide(4-HPR) in prostate cancer patients.** Urban, D., Myers, R., Manne, U., Weiss, H., Mohler, J., Poulin, N., Grubbs, C., Lieberman, R., Kelloff, G., Grizzle, W. University of Alabama at Birmingham, Birmingham, AL 35294 and National Cancer Institute, Bethesda, MD 20892.

An NCI sponsored, phase II, placebo controlled double blind randomized trial of N-(4-Hydroxyphenyl) retinamide (4-HPR) in patients with prostate cancer in a one month period prior to radical prostatectomy was carried out. Thirty three men completed the study. Biopsies of the prostate at diagnosis and surgery were compared for effects on surrogate endpoint biomarkers (SEBs). Results from 25 patients indicate significant differential expression of SEBs in pretreatment specimens of uninvolved prostatic tissue, prostatic intraepithelial neoplasia (PIN) and prostate cancer. The mean erbB-2 expression was 0.58 in uninvolved vs. 1.04 in PIN ( $p=0.002$ ); while the mean erbB-2 expression was 1.35 in prostate cancer ( $P=0.0007$ , uninvolved vs. prostate cancer). A similar pattern of increased biomarker expression between uninvolved and PIN or prostate cancer tissues was observed for EGF-receptor (mean = 1.21, 1.87, 1.76 for uninvolved, PIN and prostate cancer, respectively) and erbB-3 (mean = 0.81, 1.59, 1.30 for uninvolved, PIN and prostate cancer, respectively). There were no significant differences observed in 4-HPR vs placebo patients after treatment. Increased expression of SEBs was observed in all patients and may be caused by the diagnostic biopsy. A chemoprevention affect by 4-HPR was not demonstrated.

**#2850 The potential use of HPV copy number and E6/E7 expression as biomarkers in cervical dysplasia.** Ruffin M.T., Bailey J.M., Underwood D., Beniasz M., Gregoire L., Johnston C., Reynolds R.K., Normolle D., Reed B.D., Michael C., Gorenflo D.W., Lancaster W., Kmak D., Munkarah A.R., and Brenner D.E. University of Michigan Medical Center and Comprehensive Cancer Center, Ann Arbor, MI 48109; and Wayne State University School of Medicine, Detroit, MI 48201.

**Objective:** To determine if HPV copy number and/or expression of E6/E7 are measurable as potential biomarkers for cervical dysplasia. **Methods:** 54 women with biopsy proven CIN II or CIN III were randomized to one of three doses of all-trans-retinoic acid or a placebo agent applied topically with cervical cap for four days. Measurements were made on Day 1, Day 5, and week 12. The focus of this report is on the measurements made on Day 1 and 5. The HPV was measured from cervical smears preserved in the appropriate media. **Results:** Using stringent HPV detection methods for types 6/11, 16/18, 31/33/35, 45/52 38% of the Caucasian women did not have HPV present compared to 4% of African Americans. For these women, the biomarkers could not be measured. Among the remaining sample HPV copy number and E6/E7 could be measured. There was no discernible pattern of modulation to the dose of all trans-retinoic acid among the women with measurable biomarkers among this limited sample. **Conclusion:** Assuming that no detectable HPV type implied a low copy number and no expression of E6/E7, then these are reasonable biomarkers to pursue in cervical dysplasia. Less stringent methods for HPV detection are currently being completed.

**ACR** *American Association for Cancer Research*

# *93rd Annual Meeting*

*April 6-10, 2002 • San Francisco, California*

*Volume 43 • March 2002*

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# *Proceedings*



was identified using DGGE and characterized by sequencing (Clin Cancer Res. 2000; 6:2229). For p53, immunohistochemical staining (DO-7 monoclonal anti-body; DAKO) was considered positive if observed in 25% or more of the tumor cells. Tumors were counted as HER2 overexpressing when a strong staining of the cell membrane (c-erbB-2 antibody, DAKO) was observed in more than 10% of the tumor cells (3+ in the HerceptTest guidelines). All patients were treated according to the Danish Breast Cancer Cooperative Groups guidelines for the T2000 protocols. Results: The study included 428 patients, 205 node-negative and 223 node-positive. TP53 mutation was found in 25%, p53 expression in 48%, and HER2 overexpression in 18%. Except for null mutations, most tumors with TP53 mutations had p53 expression. However, p53 expression was also observed in 42% of TP53 wild-type tumors. TP53 mutation, HER2 overexpression, and to a lesser degree p53 expression, was associated with parameters related to tumor aggressiveness (positive lymph nodes, negative receptor status, high degree of anaplasia etc). Univariate analysis showed that disease-specific survival was correlated to tumor size, nodal status, degree of anaplasia, estrogen receptor status, TP53 mutation, p53 expression, and HER2 overexpression. When analyzed according to nodal status, TP53 mutation and HER2 overexpression, but not p53 expression, significantly correlated with poor survival probability in each of the subgroups. A Cox proportional hazard analysis including all 428 patients demonstrated that positive nodal status (1-3 positive nodes: relative risk (RR) 1.8, 95% CI: 1.0-2.7, and >3 positive nodes: RR 4.2, 2.7-6.3), TP53 mutation (RR 2.0, 1.4-3.0), and HER2 overexpression (RR 2.6, 1.8-3.8) were the only parameters which had independent poor influence on reduced disease-specific survival. TP53 and HER2 retained their independent poor influence on survival when analyzed according to nodal status. Same patterns were observed for overall survival. Conclusion: TP53 mutation and HER2 overexpression are strong markers for the prediction of disease-specific and overall survival in early breast cancer, irrespective of nodal status. p53 expression is only a weak marker, and its significance is lost when TP53 mutational analysis is included.

**#217: Correlation of established and novel molecular biology markers (E2NMBM) with long-term outcome / disease biology of stage I-III breast cancer (BrCa) patients (pts).** Multicentre collaboration of the British Columbia Breast Cancer Tissue Array Project (BCBCTAP). Joseph Ragaz, Torsten Nielsen, Marc Lippman, Mat Van de Rijn, Forrest Hsu, Stephen Chia, Angela Brodie, George Sledge, Jr., Adrian Harris, Shoukat Dedhar, Malcolm Hayes, Caroline Speers, John Spinelli, Douglas Ross, Charles Perou, David Huntsman, and Blake Gilks. BC Cancer Agency, Vancouver, BC, Canada, University of British Columbia, Vancouver, Canada, University of Michigan, Ann Arbor, MI, Stanford University, Stanford, CA, BC Cancer Agency, Vancouver, Canada, University of Maryland, Baltimore, MD, Indiana University, Indianapolis, IN, and Churchill Hospital, Oxford, UK.

Since the late 1970's, 2,154 pts participating in five British Columbia BrCa trials involving stage I-III breast cancer have been prospectively followed. Of those, 932 had their pathology material and paraffin blocks recovered, and tissue array blocks built. The following main cohorts at different risk considered to reflect different disease biology, and their 10 year overall survival (OS) rates, were identified. Also, subsets of node positive patients with extensive nodal/extracapsular spread (EN/ES+ve) with significantly worse outcome were identified (10 y OS of EN/ES+ve vs EN/ES-ve pts: 50 vs 70%,  $p < 0.001$ ). These results reflect disease of different risk and biology. In the newly started BCBCTAP, each risk category will be analyzed for expression of E2NMBM as determined by tissue array methodology. The novel markers will include those recently identified by cDNA microarray (Nature 2000, 406:747-52 MvR, DR,CP); EGF-receptor variants and ErbB1/2 (ML); integrin linked kinase and matrix metalloproteinases (SD); aromatase and the Cox2 pathway (AB); Carbonic Anhydrase & markers of hypoxia (SC, AH); p-glycoprotein / MDR, and cyclin D/Ki67/Bcl2 pathways (MH); VEGF (GS); and EMSY, a newly identified gene amplified in many breast cancers and associated with BRCA-1 (DH). Their multivariate interaction with the conventional pathology markers & the 20 year outcome of above patients, as well the proposal for more collaboration with screening of novel genetic markers on additional BrCa pts from the BCBCTAP, will be presented.

	10 year OS (%)
Node (-)	81.5%
Node (+)	58.5%
Stage III	40.1%
Inflammatory	13.8%

**#218 Expression of BP1, a homeobox gene, strongly correlates with ER expression in breast cancer.** Patricia E. Berg, Arnold Schwartz, Holly Stevenson, Gregory Davenport, Jan M. Orenstein, Peter Gutierrez, and Sidong Fu. George Washington University Medical Center, Washington, DC, Dynport Vaccine Company, LLC, Frederick, MD, and University of Maryland Medical School, Baltimore, MD.

We have cloned a potential new human oncogene termed BP1 which contains a homeobox and is a member of the Distal-less (DLX) family of homeobox genes. BP1 expression was examined in tissues from 29 newly diagnosed breast cancer patients. Overall, 66% expressed high levels of BP1 mRNA. BP1 expression was seen in 92% of the high grade, estrogen receptor (ER) negative, progesterone receptor (PR) negative cancers, but in only 40% of ER positive, PR positive breast

cancers. In contrast, BP1 was expressed at a very low level in only one of six normal breast tissues. Interestingly, all trans-retinoic acid (ATRA), a retinoid used therapeutically to treat breast cancer, was found to repress BP1 in MCF7 cells. Of potential relevance to breast cancer, BP1 maps near BRCA1. Supporting the oncogenic potential of BP1, we previously showed that BP1 mRNA is overexpressed in 63% of adult acute myeloid leukemia (AML) patients, although it is barely detectable in normal bone marrow. Ectopic expression of BP1 led to increased survival of K562 leukemia cells, while reducing BP1 expression caused apoptosis. Therefore, BP1 appears to be part of an anti-apoptotic pathway, suggesting a general mechanism by which BP1 could function as an oncogene. Our analysis of breast tumors suggests that BP1 may be a new marker in breast cancer for poor prognosis tumors and that it is a potential molecular target for therapy, an idea supported by BP1 repression by ATRA.

**#219 Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer.** Lin Wang, Robert Z. Luo, Jinsong Liu, Aysegul A. Sahin, Amanda McWatters, Robert C. Bast, Jr., and Yinhua Yu. The University of Texas M D Anderson Cancer Center, Houston, TX.

Ductal carcinoma in situ (DCIS) is an early, localized stage of multistep breast carcinogenesis that accounts for approximately 20-25% of mammographically detected breast cancers. A significant fraction of untreated DCIS will evolve into invasive cancer. ARHI is an imprinted tumor suppressor gene that is expressed in normal breast epithelial cells but that is down-regulated in a majority of breast cancers. In order to investigate the relationship of ARHI expression to the progression of breast cancer, we examined ARHI expression in 50 formalin-fixed and paraffin-embedded DCIS specimens from the Breast Cancer Tissue Bank at M. D. Anderson Cancer Center. Normal breast epithelium was found in 43 specimens and invasive breast carcinoma was found in 20 specimens. Both immunohistochemistry and in situ hybridization were used to evaluate ARHI expression. Prior to immunohistochemical assays, tissue sections were steam heated for 3 min to enhance antigen expression. After incubation with a murine monoclonal anti-ARHI antibody, ARHI was detected with biotin-streptavidin peroxidase display. Antibodies against Leukocyte Common Antigen were used as a negative control. For in situ hybridization, an ARHI mRNA antisense probe was employed to detect the ARHI gene. An ARHI mRNA sense probe was used as a negative control and the housekeeping gene GAPDH mRNA antisense probe was used as a positive control. ARHI mRNA and protein were identified in all normal breast epithelia. ARHI expression was concentrated in cytoplasm and rarely present in the nucleus. Compared to adjacent normal breast epithelia, ARHI protein expression was down-regulated in 35% (15/43) of DCIS and 65% (13/20) of invasive carcinomas. When DCIS and invasive cancer were present in the same sample, ARHI was further down-regulated in invasive carcinoma by 40% (6/20). In two cases (2/20, 10%) of invasive carcinoma, ARHI protein expression was totally lost. Consistent results were obtained with the in situ hybridization. Our study indicates that a decreased ARHI expression is associated with progression of breast cancer.

**#220 A candidate metastasis associated genetic marker for ductal mammary carcinoma.** Hui Zhao, Zuohe Fan, Lawrence Herbst, Dwayne Breining, Joan G. Jones, Panna S. Mahadevia, Harold P. Klinger, Bhadrarain Vikram, and Mohanrao P. Achary. Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY, and Albert Einstein College of Medicine and Montefiore Medical Center.

Metastasis is responsible for most deaths from breast cancer. The objective of this project is to identify a panel of molecular genetic markers for detecting those 13% of mammary carcinoma patients with negative lymph nodes that are prone to developing metastasis even after surgery, or surgery combined with radiation and/or chemotherapy. This would also benefit the remaining 87% patients, who could be treated more conservatively, sparing them the physical, mental and financial costs of the radical treatment. The long-term goal of this study is not only to find markers for prognosis but also to identify metastasis associated genes. Such knowledge could guide the development of improved therapies. The working hypothesis is that in order to achieve the metastatic state, primary mammary carcinoma cells must acquire genetic alterations in addition to those that led to transformation. In order to isolate genes associated with the suppression of metastasis (whose loss would increase the metastatic potential of the tumor), Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumors with that of the corresponding metastatic lymph node. The primary tumor and metastatic cells were recovered by laser capture microdissection and DNA samples extracted from those cells were used for RDA and for Southern blotting and PCR methods to screen metastasis associated gene sequences (MAGS). Eleven candidate MAGS were recovered that were apparently lost in metastatic cells. One of these, MAGS-IX when used to screen normal, primary and metastatic cell DNA from 3 additional breast carcinomas, was found to be lost in metastatic cell DNA of two of these samples. Thus in 3 out of 4 cases (including the original case used for RDA) MAGS-IX was apparently lost from those primary tumor cells that became metastatic. This makes MAGS-IX a potential candidate for being a metastasis suppressor gene. RH mapping localized MAGS-IX to a 21cR interval between markers, D105539 and D105549, on human chromosome 10, band q21.1. Homology searches revealed it to have 94% sequence identity to a clone on human chromosome 10 (AC022541) but not to any other known gene sequence, suggesting

## CLINICAL RESEARCH 1

that this is a novel MAGS. Additional archival cases are being screened to determine if MAGS IX is a reliable marker for identifying breast cancers that are prone to becoming metastatic.

**#221 Shc adaptor proteins in breast cancer prognosis: Novel molecular markers that predict aggressive Stage 1 tumors.** Pamela A. Davol, Robert Bagdasaryan, and A. Raymond Frackelton, Jr. *Roger Williams Medical Center, Providence, RI.*

In a 12-year, retrospective study of clinical outcome (with at least a 5 year follow-up) of breast cancer patients in the Roger Williams Cancer Center database and tumor registry, a 10% mortality associated with disease recurrence was observed in patients diagnosed with Stage 1 breast cancer ( $n = 212$  patients). Accordingly, there is an evident need for molecular markers that may differentiate aggressive, early-stage breast cancers from less invasive lesions and thus guide surgical and adjuvant treatment options. Immunohistochemical staining of phosphorylated Shc (PY-Shc: an activated, adaptor protein that facilitates tyrosine kinase signaling and tumorigenesis) and p66-Shc (a Shc isoform that inhibits this signaling cascade) in 98 archival, formalin-fixed, diagnostic breast tumor biopsies (Stage 0 to Stage 4 patients) demonstrated a positive linear correlation between the ratio of PY-Shc to p66-Shc staining intensity in regard to patient stage ( $r = 0.4$ ;  $p < 0.0001$ ); with high PY-Shc/low p66-Shc corresponding to advanced disease stage at the time of diagnosis. When the PY-Shc to p66-Shc ratio was analyzed in primary tumors from Stage 1 breast cancer patients and then retrospectively compared to patient outcome ( $> 5$  yr follow-up), the ratio for randomly selected tumors from patients with no disease recurrence ( $0.66 \pm 0.03$ ;  $n = 30$ ) was significantly lower compared to patients with disease recurrence ( $0.90 \pm 0.07$ ;  $n = 8$ ) ( $p < 0.005$ ). These studies suggest that the PY-Shc to p66-Shc ratio may serve as a viable prognostic marker for identifying aggressive, early stage breast cancers. (Supported by Department of Defense Breast Cancer Grants: BC980415 and DAMD17-99-1-9363)

**#222 Quantitative gene expression of human anterior gradient (hAG-2R) in human breast tumor tissue and its potential as a prognostic indicator in breast cancer.** Monica Madden Reinholz, Stephen J. Iturria, Patrick C. Roche, and Judith S. Kaur. *Mayo Clinic, Rochester, MN.*

The molecular basis for the observed differences between estrogen receptor-positive (ER+) and ER-negative (ER-) breast tumors remains unclear. The human homologue of the *Xenopus laevis* cement gland gene *Xenopus Anterior Gradient-2* (XAG-2), hAG-2R, was previously found to be co-expressed with ER in breast cancer cell lines. Because ER is probably only one of a set of expressed genes that are responsible for the phenotype of hormone-responsive breast cancer, we examined the gene expression pattern of hAG-2R in different stage breast tumor tissues. In the present study, we used reverse transcription (RT) and fluorescence-based kinetic PCR (Taqman) to determine the mRNA levels of hAG-2R in normal breast tissues, ductal carcinoma in situ (DCIS) tissues, primary breast tumors, and distant breast metastatic tissues. We observed that the average hAG-2R gene expression significantly increased over seven-fold ( $p < 0.0005$ ) in five DCIS tissues and over nine-fold ( $p < 0.0006$ ) in 24 primary breast tumor tissues compared to the average hAG-2R gene expression from 18 normal breast tissues. Eighty percent of the five immunohistochemically detected ER+ DCIS samples overexpressed hAG-2R (overexpression defined as  $> 2$  standard deviations above the mean expression of normal breast tissue). Seventy-four percent of the 19 ER+ primary tumors overexpressed hAG-2R, and only one of the five ER-tumors overexpressed hAG-2R. The average hAG-2R gene expression decreased over 17-fold ( $p < 0.004$ ) in three breast liver metastasis compared to normal breast tissue. In addition, hAG-2R gene expression increased over 45-fold in breast cancer metastatic to bone and was not changed in breast cancer metastatic to ovary compared to normal breast tissue. In a separate panel of 35 node negative breast tumor tissues, 90% of 20 good outcome (indicated by no disease recurrence at five years) tumors overexpressed hAG-2R and 40% of 15 bad outcome (indicated by disease recurrence at less than three years) tumors overexpressed hAG-2R. The average hAG-2R gene expression was four-fold higher in the good outcome tumors compared to the bad outcome tumors. These results demonstrated significant differential gene expression of hAG-2R in different stage breast cancer tissues and the co-expression of hAG-2R and ER in DCIS and primary breast tumor tissues. These results suggest that in addition to ER, hAG-2R may be another gene responsible for the phenotype of hormone-responsive breast cancer. Lastly, hAG-2R gene expression may prove to be a useful prognostic indicator for breast cancer. This work was supported by the DOD grant DAMD-17-00-1-0633, the Mayo Foundation, and the Breast Cancer Research Foundation.

**#223 Nipple fluid basic fibroblast growth factor in breast patients.** Zhi-Ming Shao, Zhen-Zhou Shen, Liping Zhang, Maryam Sartippour, Canhui Liu, He-Jing Wang, Robert Elashoff, Helena Chang, and Mai Nguyen. *University of California, Los Angeles, Los Angeles, CA, and Fudan University, Shanghai, China.*

Purpose: It has been shown that early detection of breast cancer saves lives. Recently, there has been increasing interest in nipple fluid as a potential avenue for breast cancer diagnosis. Experimental Design: In this study, we measured the levels of an angiogenic factor bFGF (basic fibroblast growth factor) in the nipple fluid of healthy subjects as well as patients with benign breast conditions, those at high risk for breast cancer, and patients with ongoing breast cancer. Results:

We found that high risk breasts ( $562 \pm 755$  pg/ml,  $p = 0.009$ ) and cancerous breasts ( $870 \pm 1,848$  pg/ml,  $p = 0.001$ ) produced higher levels of bFGF in nipple fluid in comparison to benign breasts ( $134 \pm 401$  pg/ml). With a cutoff level of 150 pg/ml of bFGF, sensitivity was calculated to be 75%, specificity 84%, and the correct diagnostic rate 82%. Conclusions: We conclude that nipple fluid bFGF may be useful in the diagnosis of breast cancer, and deserves further studies.

**#224 High-level amplification of C-MYC is associated with progression from the *in situ* to the invasive stage of breast carcinomas.** Els C. Robanus-Maandag, Cathy A. J. Bosch, Petra M. Kristel, Augustinus A. M. Hart, Ian F. Faneyte, Petra M. Nederlof, Johannes L. Peterse, and Marc J. van de Vijver. *The Netherlands Cancer Institute, Amsterdam, Netherlands.*

Carcinoma *in situ* of the breast is believed to be a genetically advanced precursor lesion for invasive carcinoma, since *in situ* lesions already demonstrate genomic changes found in invasive lesions. However, no specific genetic alterations have been identified so far that are associated with progression from the *in situ* to the invasive stage. As most invasive carcinomas also contain an *in situ* component, we compared the genetic alterations in the *in situ* and invasive component of the same tumor. Of 12 invasive breast carcinomas, we microdissected the invasive and adjacent *in situ* component, isolated DNA and performed comparative genomic hybridization. In some tumors, we observed a few distinct differences between otherwise identical genome profiles of both components suggesting that the number of genetic alterations involved in breast tumor progression is limited. Further analysis of such a difference in one tumor by fluorescence *in situ* hybridization (FISH) revealed high-level amplification of C-MYC in the invasive component only. To investigate the frequency of this correlation, we identified from a panel of 188 invasive carcinomas 18 cases with C-MYC amplification, 9 of which with an adjacent *in situ* component. Using FISH, more than 5 C-MYC signals per nucleus were found in 7 and C-MYC/CEP8 ratios  $> 4$  were found in 5 of 9 invasive components but not in any associated *in situ* component. With probes of 3 BAC clones derived from chromosome 8q the minimal amplified region in this set of C-MYC-amplified tumors was defined at 8q24.1-8qter. C-MYC amplification was correlated with overexpression of C-MYC and two of its target genes, *TERT* and *FBL*. Thus, high-level C-MYC amplification is the first identified genetic alteration that is strongly associated with progression from the *in situ* to the invasive stage of breast carcinomas.

**#225 Hsp27, angiogenesis and cadherins in human breast cancer biopsy samples.** Mariel A. Fanelli, F. Dario Cuello Carrion, Francisco E. Gago, Olga Tello, and Daniel R. Ciocca. *Institute of Experimental Medicine and Biology of Cuyo (IMBECU), Mendoza, Argentina, and School of Medicine, National University of Cuyo (UNC), Mendoza, Argentina.*

Breast cancer is a heterogeneous disease and the correct identification of the patients who will have a poor prognosis is of clinical value to provide the best treatment options and to plan the follow-up. There are several pathological and molecular prognostic factors and it is clear that the combination of several of them will be necessary to discover the patients with poor prognosis, e.g. those developing distant metastases. In the present study we have evaluated the prognostic significance of hsp27 in the blood vessels of breast cancer patients correlating its expression with that of: a) coagulating factor VIII (FVIII, used to measure angiogenesis), and b) cadherins (E-cadherin and P-cadherin) and beta Catenin. Cadherins are important molecules involved in cell-cell adhesion, some of them have been related with the prognosis of breast cancer. The study involved 113 patients, 76 with a median follow-up of 5 years. The breast cancer biopsy samples were processed for immunohistochemistry. Hsp27 could be detected in the endothelium of small blood vessels as well as in the tumor cells, the number of hsp27-positive vessels was higher in the tumor areas with infiltrating lymphocytes. There was no correlation between the presence of hsp27-positive blood vessels and the amount of blood vessels positive for FVIII, FVIII was a better marker for angiogenesis. The expression of hsp27 in the blood vessels did not correlate with the development of distant metastasis, however, angiogenesis (FVIII) correlated with poor prognosis ( $p(0.02)$ ). Tumors expressing P-cadherin showed more hsp27-negative blood vessels ( $p(0.05)$ ). The presence of P-cadherin (but not E-cadherin) in the membrane of tumor cells was associated with poor prognosis ( $p(0.02)$ ). In tumor cells, hsp27 did not correlate with P-cadherin expression. Beta catenin content did not correlate with P-cadherin expression with disease prognosis. In summary, poor prognosis was seen in patients with P-cadherin expression and with elevated angiogenesis.

**#226 Lipophilin B, lipophilin C, and ECM1, a new member of the uteroglobin family, are overexpressed in endometrial and breast cancer.** Susana Salceda, Anton Nguyen, Carey Drumright, Andrei Munteanu, Melinda Au, Charis Lawrenson, Nam W. Kim, and Roberto A. Macina. *diaDex, Inc., South San Francisco, CA.*

The members of the uteroglobin family are small, secretory proteins, whose physiological functions remain unclear. Using cDNA databases mining, we have identified a new member of the human uteroglobin family, which we have designated ECM1 (Endometrial Cancer Marker 1). Alignment with data from the Human Genome Project, showed that ECM1 is located on chromosome 11 as has been described for other members of this family. ECM1 protein has 90 amino acids, with a predicted signal peptide in its amino terminal. It shares 61% similarity with lipophilin B, 34% with mammaglobin, and 31% with lipophilin C. Analysis of

## Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression

M.P. Achary,<sup>a</sup> W. Jaggernauth,<sup>a</sup> E. Gross,<sup>a</sup> A. Alfieri,<sup>b</sup> H.P. Klinger<sup>c</sup> and B. Vikram<sup>a</sup>

<sup>a</sup>Department of Radiation Oncology, Albert Einstein College of Medicine of Yeshiva University, and Montefiore Medical Center, Bronx; <sup>b</sup>Department of Radiation Oncology, Beth Israel Medical Center, New York;

<sup>c</sup>Department of Molecular Genetics, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY (USA)

Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

**Abstract.** Combining chemotherapy with radiotherapy has improved the cure rate among patients with cancers of the cervix. Although one-half to two-thirds of the patients can be cured by radiation alone, such patients cannot be identified at present and must therefore suffer the burden of chemotherapy. Our long-range goal is to identify those cervical cancers that are radiosensitive and could be cured by radiotherapy alone. The advent of methods that permit the simultaneous analysis of expression patterns of thousands of genes, make it feasible to attempt to identify the molecular events related to radiosensitivity and the associated regulatory pathways. We hypothesize that the sensitivity of tumor cells to ionizing radiation (IR) is determined by the level of expression of specific genes that may be identified with the aid of cDNA microarrays. As the first step in testing this hypothesis, we determined the gene expression differences between two cell lines exhibiting different degrees of radiosensitivity. These were derived from the same tumor prior to treatment from a patient with squamous cell carcinoma of the cervix. The mRNA from these cells was subjected to cDNA analysis on a microarray of 5,776 known genes

and ESTs. The expression of 52 genes of the total of 5,776 was elevated (maximum 4.1 fold) in the radioresistant cells as compared to the radiosensitive cells. Ten of the 52 sequences are known genes while 42 are ESTs. Conversely, the expression of 18 genes was elevated in the sensitive cells as compared to the resistant cells. Seven of these 18 are known genes while eleven are ESTs. Among the genes expressed differentially between the resistant and sensitive cells were several known to be associated with response to IR and many more genes and ESTs that had not previously been reported to be related to radiosensitivity. The genes that showed the greatest overexpression in the radioresistant cell line were metal-regulatory transcription factor-1, cytochrome P450 CYP1B1, adenomatous polyposis coli, translation elongation factor-1, cytochrome-c oxidase, whereas in the sensitive cell line, transcription factor NF-kappa-B, metalloproteinase inhibitor-1 precursor, superoxide dismutase-2, insulin-like growth factor binding protein-3, guanine nucleotide-binding protein and transforming growth factor beta-induced protein were overexpressed.

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Request reprints from Dr. Mohan P. Achary, Ullman-1219, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 (USA); telephone: 718 430 2699; fax: 718 430 2454; e-mail: achary@aecom.yu.edu

Combining chemotherapy with radiotherapy has improved the survival rates of patients with cervical cancers (Keys et al., 1999; Morris et al., 1999; Rose et al., 1999; Whitney et al., 1999). Although one-half to two-thirds of the patients can be cured by radiation therapy alone and do not need chemotherapy, these cannot now be identified so that such patients must unnecessarily suffer the toxicity and the expense of chemother-



apy. The advent of microarray gene expression technology permits the simultaneous analysis of the levels of expression of thousands of genes. Thus, the study of molecular genetic events that are related to radiosensitivity can be examined. This may also lead to identifying genes and gene regulatory pathways related to the resistance of cells to therapeutic procedure. One of our long-range goals is to use this technology to identify those cancers that are radiosensitive and can thus be cured by radiotherapy alone. Another goal is to identify those cancers that are not controlled by the combined therapy and thus hopefully identify molecular targets for the development of therapeutic strategies.

We hypothesize that the sensitivity of tumor cells to ionizing radiation (IR) is dependent on alterations in the expression of specific genes. As the first step in testing this hypothesis, we determined the differences in the gene expression profiles of two cervical cancer cell lines derived from the same tumor but exhibiting very different degrees of radiosensitivity. We present the results in this report.

## Materials and methods

### Cell culture

Several cervical cancer cell lines that were derived prior to treatment by punch biopsies from patients with cervical cancers were kindly provided to us by Dr. Richard A. Britten of Cross Cancer Institute, Edmonton, Canada. These were in the fourth to fifth passage. For this report we used one pair of cell lines derived from the same tumor (HT137). These cell lines were cultured in the same way as described by Allalunis-Turner et al. (1991) and Britten et al. (1996). Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and antibiotics was used. The cells were subcultured every 4–5 days to ensure exponential growth.

### Clonogenic cell survival

Following the procedures of Britten et al. (1996) clonogenic survival tests were performed. Briefly, cells were plated in 100-mm petri dishes at known densities and after 4–6 hr the cells were exposed to 2 Gy of radiation using a linear accelerator (Clinac 6-100, Varian oncology systems, Palo Alto CA). After 2 weeks the surviving colonies were stained with crystal violet solution and stained colonies containing more than 50 cells were counted. The surviving fraction (SF) after exposure to 2 Gy for the HT137R cells was thereby determined to be 0.67, and 0.35 for the HT137S cells.

### Microarray sample preparation

The cDNA microarray chips and the image scanning programs were developed in the Genome Microarray Facility of the Albert Einstein College of Medicine. The human cDNA microarray chips used in this study each contain 5,776 cDNA sequences representing arbitrarily selected known genes, housekeeping genes and ESTs. The cDNA sample from the radiation sensitive cell line HT137S was labeled with the fluorescent dye, Cy5 (red) and that of the resistant cell line HT137R was labeled with Cy3 (green). A customized ScanAlyse program (Eisen et al., 1998) was used for post-acquisition processing and for database mining functions. The fluorescent signals representing hybridization to each arrayed sequence were analyzed to determine the relative amount of mRNA that hybridized with each sequence in both samples. Full details of the procedure are given on our website: <http://sequence.aecom.yu.edu/bioinf/funcgenomic.html>.

### Synthesis of labeled cDNA probe

One hundred micrograms of total RNA each were isolated from the HT137S and HT137R cell pellets using the Qiagen RNeasy extraction kit. The RNA samples were incubated separately with Oligo dT12–18 at 65°C for annealing of oligo primers. Two mixtures were prepared, one containing first strand buffer, DTT low dNTP mix, RNasin and the fluorochrome Cy3 for HT137R cells. The second mixture was the same except that Cy5 was used for the HT137S cells. To these mixtures reverse transcriptase (RT, BRL

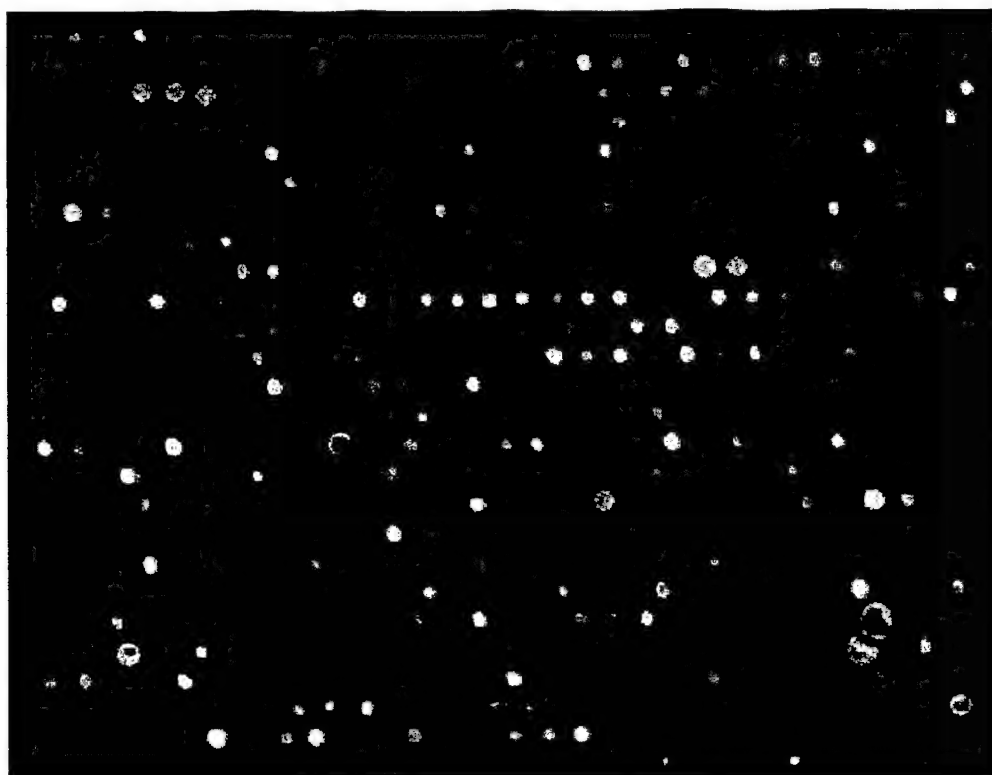
**Table 1.** Fifty-two genes and ESTs (out of the total 5,776) whose expression was elevated at least two-fold in the HT137R (Resistant) cells compared with the HT137S (Sensitive) cells

GB Accession number	Description of genes/ESTs
T72724	EST
T80917	EST
R79518	EST
H83358	EST
N42169	EST
N43977	EST
W90242	EST
AA004354	EST
AA004921	EST
AA004570	EST
AA005086	EST
AA010280	EST
AA203495	Metal-regulatory transcription factor-1
H21756	EST
H06460	EST
W02900	Cytochrome P450 CYP1B1, dioxin-inducible
N90485	EST
R00760	EST
R23082	EST
R33908	EST
N28450	EST
H29191	Adenomatous polyposis coli, alt. Splice-1
T83093	EST
R69208	EST
H82175	EST
N33565	EST
T99685	EST
R31339	EST
R37928	EST
H20450	EST
H40309	EST
N49030	EST
N36501	Phosphodiesterase
N28330	Glycoprotein MUC18, alt. Splice-2
N28369	EST
N78414	EST
T79703	EST
T85390	EST
T86312	EST
T86315	Neurotoxin, eosinophil-derived
T87438	EST
AA190599	Translation elongation factor-1, gamma
H83614	EST
AA028123	EST
H21167	EST
H46937	EST
R83166	EST
R92654	EST
N31224	Glycoprotein MUC18, alt. Splice-3
N99222	EST
H52746	Cytochrome c oxidase, IV subunit
AA146629	Catenin, alpha 2(E), alt. Splice-1

Superscript II) was added and incubated at 42°C for 2 h. Then, to inactivate the RT, the tubes were heated at 94°C. The volume of this mixture was increased to 100 µl by adding buffered RNase 1 and incubated at 37°C to digest any RNA not converted to cDNA. Then the contents of both the tubes were mixed and passed through Microcon YM 50 retention columns. The probe was collected and the volume adjusted to 6.5 µl.

### Array slide preparation

The microarrays on slides were vapor moistened and quickly exposed to 200 mJ total energy in a UV Stratalinker. The slides were moistened again over boiling water and quickly dried on a hot plate. They were then treated with succinic anhydride solution for 15 min, rinsed in 0.1% SDS followed by water, and placed in a 95°C water bath for 3–5 min after which they were dipped into ice-cold ethanol. Excess ethanol was removed from the slides by spinning the rack of slides at 500 rpm.



**Fig. 1.** A portion of the ScanAlyze picture from the cDNA microarray. The green spots represent genes whose expression is two-fold or greater in the HT137R cells than in the HT137S cells. The red spots represent genes whose expression is two-fold or greater in the HT137S cells. The yellow spots represent genes that are nearly equally expressed in both types of cells. The violet spots are "flagged" spots, i.e. those with dust/specks or similar artifacts. These are flagged to avoid their inclusion in the analysis. Circular customized grids covering each of the 5,776 spots are superimposed on the ScanAlyze spots, to assure the import of the correct color intensity of each spot area into an Excel file for data analysis.

#### *Prehybridization of slide and probe*

Prehybridization solution (20  $\mu$ l, containing formamide, SSPE, SDS, Denhardt's solution and salmon sperm DNA) was placed on the array and covered with a coverglass. The slides were placed in a chamber and prehybridized at 50°C for one hr. Simultaneously the probe was prehybridized at 50°C for 1 h. For prehybridization the 6.5- $\mu$ l probe was mixed with blocking solution (human cot-1 DNA, SDS, Denhardt's and SSPE solution) to bring the volume to 20  $\mu$ l. It was then heated, centrifuged and incubated as described above. After 1 h both the slides and the probe were ready for hybridization.

#### *Hybridization and washing the slides*

The prehybridized probe (20  $\mu$ l) was dropped on the array region of each slide and a fresh coverslip was placed over the slide. Hybridization was performed overnight at 50°C.

For washing the slides were placed in a glass slide holder containing 1 $\times$  SSC and 0.1% SDS at room temperature, where the coverslips fell off. The slides were then removed and placed in another slide holder containing 500 ml of 0.2% SSC and 0.1% SDS for 15 min. The slides were then transferred to another chamber, containing 0.2 $\times$  SSC for 20 min, after which they were ready for scanning.

#### *Analysis of microarray results*

The hybridization signals were scanned with a laser confocal scanner which generates 2-color TIF images. Scans for the two fluorescent probes were normalized to the fluorescence intensity of beta actin and GPDH (Hel-

ler et al., 1997) and the ratios of the fluorescence intensities of all the spots was determined. Intensities for each spot in each channel were calculated after subtraction of the background. Background "noise" was reduced by using a 2-standard deviation cutoff on all expression values in order to identify only those genes with significantly different expression (Chen et al., 1997; Eisen et al., 1998; Amundson et al., 1999; Duggen et al., 1999; Lee et al., 1999; Pollack et al., 1999). Red spots represent genes whose expression in the HT137S cells is at least double that of the HT137R cells. Green spots represent those genes whose expression was double in the HT137R cells as compared to the HT137S cells, whereas yellow spots represent genes whose expression was similar in both the cell lines (Fig. 1). Genes and ESTs had to be expressed at similar fluorescent intensity ratios in at least two microarray hybridizations to be included in the analysis.

## **Results**

The expression of 52 genes (0.9%) out of the total 5,776 was elevated (2–4.1 fold) in the HT137R cells as compared to the HT137S cells (Table 1). Ten of these 52 are known genes, while 42 are ESTs. Conversely, the expression of 18 genes was elevated 2–2.9 fold in the HT137S cells compared with the HT137R cells (Table 2). Seven of these 18 are known genes while 11 are ESTs. The genes that showed the greatest overex-

**Table 2.** Eighteen genes (out of the total 5,776) whose expression was elevated at least two-fold, in the HT137S (Sensitive) cells compared with the HT137R (Resistant) cells

GB Accession number	Description of genes/ESTs
R86053	Transcription factor NF-kappa-B
R97630	Alcohol dehydrogenase-1, class 1, alpha polypeptide
N67954	EST
R78823	EST
AA001324	EST
T99143	EST
N57354	EST
AA143155	Superoxide dismutase 2, mitochondrial, alt. Splice-1
R75975	Monocyte chemotactic protein -1
R25247	EST
R38114	EST
R80595	EST
N31417	Insulin-like growth factor binding protein 3
N28758	EST
R78657	Guanine nucleotide-binding protein HM89
N42864	EST
AA002125	EST
AA037281	Transforming growth factor beta-induced protein

pression in the radioresistant cell line were metal-regulatory transcription factor-1, cytochrome P450 CYP1B1, adenomatous polyposis coli, translation elongation factor-1 and cytochrome-c oxidase, whereas in the sensitive cell line, transcription factor NF-kappa-B, metalloproteinase inhibitor-1 precursor, superoxide dismutase-2, insulin-like growth factor-binding protein-3, guanine nucleotide-binding protein and transforming growth factor beta-induced protein were overexpressed.

## Discussion

It is clear from the results that cell lines from the same tumor but with different radiosensitivities exhibit different patterns of gene expression. Having determined that such differences exist the next question to be answered is to what extent are these gene expression alterations related to radiosensitivity. We hope to be able to answer this question by examining a larger series of similar cell lines and also radiosensitive and radioresistant primary cervical carcinomas. If the same specific genes have altered expression in many different cases then this would be evidence that they are related to the cell's response to radiation. It is encouraging that among the genes expressed differentially between the resistant and sensitive cells in this study were

several that are known to be associated with the cell's response to IR. These are: transcription factor NF-kappa-B, superoxide dismutase-2, insulin-like growth factor-binding protein-3, guanine nucleotide-binding protein, and transforming growth factor beta-induced protein (Arnold et al., 1999; Kawai et al., 1999; Epperly et al., 2000; Kuninaka et al., 2000; Williams et al., 2000). As noted there were also a number of genes and ESTs which have previously not been reported to be related to radiosensitivity (Tables 1 and 2). Their importance in conferring the radioresponse phenotype to a cell will clearly require much more extensive studies, particularly because the phenomenon appears to be fairly complex involving several genes and gene pathways. As an example, IR-induced patterns of gene expression may vary according to the cellular context as demonstrated by Amundson et al. (1999) who studied IR-induced gene expression in human myeloid ML-1 cells using a microarray consisting of 1,238 gene sequences. They found that 48 sequences (including 30 not previously identified as IR-responsive) were significantly influenced by IR. Induction by IR of a subset of these genes was examined in a panel of 12 human cell lines, and it was observed that the responses varied widely in cells from different tissues of origin and different genetic backgrounds (Amundson et al., 1999).

Like many other investigators we have regarded as significant only those genes whose expression was altered by at least a factor of two. However, we recognize that this cutoff point is arbitrary and that there may be important genes involved whose expression was altered by less than a factor of two. Another limitation of this study is that the microarray utilized consisted of only 5,776 arbitrarily selected known genes, house-keeping genes, and ESTs. This limitation can now be overcome since microarrays with much larger numbers of genes are now available. In addition, customized arrays are becoming available with genes known to, or suspected of, participating in the process under study. An example of the successful application of the latter approach is the recent demonstration with a "lymphochip" that large-cell lymphomas responding well to CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy could be distinguished from those responding poorly (Alizadeh et al., 2000).

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# Altered Gene Expression Pattern in Cultured Human Breast Cancer Cells Treated with Hepatocyte Growth Factor/Scatter Factor in the Setting of DNA Damage<sup>1</sup>

Ren-qi Yuan, Saijun Fan, Mohan Achary, Donn M. Stewart, Itzhak D. Goldberg, and Eliot M. Rosen<sup>2</sup>

Department of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, New Hyde Park, New York 11040 [R.-q. Y., S. F., I. D. G., E. M. R.]; Departments of Radiation Oncology [M. A., E. M. R.] and Developmental and Molecular Biology [E. M. R.], Albert Einstein College of Medicine, Bronx, New York 10461; and Metabolism Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892 [D. M. S.]

## ABSTRACT

The cytokine hepatocyte growth factor/scatter factor (HGF/SF) protects epithelial and cancer cells against DNA-damaging agents via a pathway involving signaling from c-Met → phosphatidylinositol-3-kinase → c-Akt. However, the downstream alterations in gene expression resulting from this pathway have not been established. On the basis of cDNA microarray and semiquantitative RT-PCR assays, we found that MDA-MB-453 human breast cancer cells preincubated with HGF/SF and then exposed to Adriamycin (ADR), a DNA topoisomerase II inhibitor, exhibit an altered pattern of gene expression, as compared with cells treated with ADR only. [HGF/SF+ADR]-treated cells showed altered expression of genes involved in the DNA damage response, cell cycle regulation, signal transduction, metabolism, and development. Some of these alterations suggest mechanisms by which HGF/SF may exert its protective activity, e.g., up-regulation of polycystic kidney disease-1 (a survival-promoting component of cadherin-catenin complexes), down-regulation of 51C (an inositol polyphosphate-5-phosphatase), and down-regulation of TOPBP1 (a topoisomerase II binding protein). We showed that enforced expression of the cdc42-interacting protein CIP4, a cytoskeleton-associated protein for which expression was decreased in [HGF/SF+ADR]-treated cells, inhibited HGF/SF-mediated protection against ADR. The cDNA microarray approach may open up new avenues for investigation of the DNA damage response and its regulation by HGF/SF.

## INTRODUCTION

The cytokine HGF/SF<sup>3</sup> is a pleiotrophic mediator of multiple biological functions that plays significant roles in embryonic development, tissue and organ repair, tumorigenesis, and angiogenesis. HGF/SF has been found to protect various cell types against apoptosis induced by a variety of stimuli, including loss of contact with the substratum (1), exposure to staurosporine (a protein kinase inhibitor; Refs. 2, 3), and DNA damage (4-7). We have reported previously that various epithelial and carcinoma cell lines are protected by HGF/SF against apoptotic cell deaths induced by DNA-damaging agents, including ionizing radiation, ultraviolet (UV-C) radiation, and ADR (also known as doxorubicin; Ref. 5). ADR is a DNA intercalator and a DNA topoisomerase II $\alpha$  inhibitor that induces single- and double-strand DNA breaks similar to those induced by ionizing radiation.

Interestingly, preincubation with HGF/SF also reduced the number of residual DNA strand breaks at 24 h after exposure to ADR or ionizing radiation, suggesting that HGF/SF may also enhance the rate of DNA repair (i.e., strand rejoining; Ref. 6). The increased DNA

repair and the cell protection against DNA damage appeared to be attributable to at least in part, to: (a) activation of a cell survival pathway involving PI3K and c-Akt (protein kinase B); and (b) subsequent stabilization of the protein levels of the antiapoptotic mitochondrial pore-forming protein Bcl-X<sub>L</sub> (5, 6).

These studies have not revealed the downstream effector genes that mediate cytoprotection by HGF/SF. Cytoprotection by HGF/SF might involve nonnuclear events, such as inactivation of proapoptotic effectors (e.g., Bad and caspase-9) by c-Akt-mediated protein phosphorylation events (8, 9). However, it might also involve prolonged patterns of altered gene expression induced by HGF/SF in the DNA-damaged cells. The latter possibility was suggested by the observation that maximal protection required a preincubation of cells with HGF/SF for at least 48 h before exposure to ADR (5). Shorter preincubation periods yielded less protection, and application of HGF/SF only at the time of ADR treatment and during the 72-h postincubation period gave no protection.

To investigate the potential alterations of gene expression that might contribute to HGF/SF-mediated cell protection, we have used a cDNA microarray approach, using a previously studied model for HGF/SF protection (5). MDA-MB-453 human breast cancer cells were preincubated with HGF/SF, exposed to ADR, and then postincubated in ADR-free culture medium for 72 h to allow the repair processes to proceed. Alterations of mRNA expression were examined in cells treated with [HGF/SF+ADR], in comparison with cells treated with ADR alone.

## MATERIALS AND METHODS

**Sources of Reagents and Vectors and Sources of Reagents and Antibodies.** Recombinant human two-chain HGF/SF was generously provided by Dr. Ralph Schwall (Department of Endocrine Research, Genentech, Inc., South San Francisco, CA). ADR (doxorubicin hydrochloride) and MTT dye (thioazyl blue) were purchased from Sigma Chemical Co. (St. Louis, MO). Expression vectors encoding full-length and truncated or deleted forms of human CIP4 have been described earlier (10). These CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an NH<sub>2</sub>-terminal myc epitope tag.

**Cell Lines and Culture.** MDA-MB-453 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with FCS (5% v/v), nonessential amino acids (100 mM), L-glutamine (5 mM), streptomycin (100  $\mu$ g/ml), and penicillin (100 units/ml; all from BioWhittaker, Walkersville, MD). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

**ADR Treatment.** Subconfluent proliferating cells in 100-mm plastic dishes or 96-well plates were preincubated in the absence or presence of HGF/SF (100 ng/ml  $\times$  48 h) in serum-free DMEM and then sham-treated (control) or treated with ADR (10  $\mu$ M  $\times$  2 h, at 37°C) in complete culture medium (DMEM plus 5% FCS). Cultures were then washed three times to remove the ADR and postincubated in fresh drug-free complete culture medium at 37°C for 72 h (again in the absence or presence of HGF/SF, respectively). Cultures were then harvested for isolation of total cell RNA and cDNA microarray or semiquantitative RT-PCR analyses.

**Transient Transfections.** Subconfluent proliferating cells were transfected overnight using Lipofectamine (Life Technologies, Inc., Rockville, MD; 10  $\mu$ g of plasmid DNA/100-mm dish) and then washed to remove the excess vector

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<sup>2</sup> To whom requests for reprints should be addressed, at Department of Radiation Oncology, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, 270-05 76th Avenue, New Hyde Park, NY 11040. Phone: (718) 470-7386; Fax: (718) 470-9756; E-mail: crosen@lij.edu.

<sup>3</sup> The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; ADR, Adriamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IP, immunoprecipitation; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR.

## Differential gene expression associated with tumorigenicity of cultured green turtle fibropapilloma-derived fibroblasts

Lawrence H. Herbst<sup>a,\*</sup>, Ratna Chakrabarti<sup>b</sup>, Paul A. Klein<sup>c</sup>, Mohan Achary<sup>d</sup>

<sup>a</sup>Department of Pathology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

<sup>b</sup>Department of Molecular Biology and Microbiology, University of Central Florida, Orlando, FL 32816, USA

<sup>c</sup>Department of Pathology Immunology and Laboratory Medicine, University of Florida, Gainesville, FL 32610, USA

<sup>d</sup>Department of Radiation Oncology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

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### Abstract

Fibroblast cell lines derived from normal skin and experimentally induced fibropapillomas of green turtles (*Chelonia mydas*), were propagated in vitro and tested for tumorigenicity in immunodeficient mice. Differential display RT-PCR was used to identify differences in messenger RNA expression between normal and tumorigenic fibropapillomatosis (FP)-derived fibroblasts from the same individual. Four unique products that were apparently overexpressed in FP and three that were apparently underexpressed were cloned and sequenced. Differential expression was confirmed for three products by Northern blotting. Two overexpressed products showed extensive sequence matches to the known mammalian cellular genes, beta-hexosaminidase and chain termination factor. The product that was underexpressed in FP showed homology with mammalian thrombospondin, a known tumor-suppressor gene and an inhibitor of angiogenesis. All of the partial gene sequences identified are novel and will require full length cDNA sequencing to further analyze their identities. These results, however, provide the foundation for further investigation to determine the role of each of these gene products in FP pathogenesis and cellular transformation. The potential for some of these products to serve as biomarkers for FP is discussed. © 2001 Elsevier Science Inc. All rights reserved.

### 1. Introduction

The green turtle, *Chelonia mydas*, is an endangered species. In the past two decades, populations of green turtles around the world have been affected by an increasing prevalence of fibropapillomatosis (FP), a disease that is characterized by multiple cutaneous and occasional visceral fibromas or fibrosarcomas [1]. This disease kills a considerable proportion of severely affected turtles and in the rest it increases the susceptibility to other mortality factors such as predation.

A major histologic feature of FP in all tissues is the proliferation of stromal fibroblasts, which suggests that fibroblasts are the cells that have undergone pathological changes [2]. Transmission experiments have implicated a viral agent as the cause of FP [3,4], but the mechanism is unknown. Possible mechanisms include either direct transformation of infected fibroblasts (neoplasia), or paracrine

stimulation of uninfected fibroblasts (hyperplasia) by another cell type that is infected or transformed by the agent. These tumor fibroblasts are well differentiated and have normal cytologic features and are morphologically indistinguishable from normal dermal fibroblasts and have similar growth patterns and serum dependence in vitro [5]. This has made comparative studies of these cells difficult, because to elucidate the molecular basis of FP fibroblast proliferation, these cells should be differentiated from normal fibroblasts. However, we have shown that FP-derived fibroblasts are tumorigenic whereas normal dermal fibroblasts are not, using an immunodeficient mouse model [5].

The purpose of this study was to begin to elucidate the molecular mechanisms of FP pathogenesis by searching for differences in gene expression between closely matched sets of tumorigenic FP-derived and non-tumorigenic normal dermal fibroblast cell lines using differential message display analysis [6]. This is a sensitive technique to identify both novel viral genes that may be differentially expressed in infected or transformed cells and also host genes whose expression is altered by infection with the FP agent or any other transforming events.

\* Corresponding author. Tel.: 718-430-8553; fax: 718-430-8556.

E-mail address: herbst@accorn.yu.edu (L.H. Herbst).



## 2. Materials and methods

### 2.1. Cell lines

Pairs of matched early passage tumor and normal skin-derived fibroblast lines derived from green turtles with experimentally induced FP were propagated at 30°C in a 5% CO<sub>2</sub> atmosphere in Dulbecco Modified Eagle Media (DMEM)/F12 supplemented with 10% FBS (GIBCO, Grand Island, NY, USA) as described previously [5]. Cultures were expanded to approximately 6×10<sup>7</sup> cells. Cells were grown to confluence and then harvested with 0.25% trypsin-1 mM EDTA in Hanks' Balanced Salt Solution (HBSS). The cells were washed in HBSS and approximately 5×10<sup>7</sup> cells were used for DNA and RNA extractions, and 1–5×10<sup>6</sup> cells were used in tumorigenicity assays to confirm their phenotype. The remaining cells were cryopreserved for further investigation.

### 2.2. In vivo tumorigenicity

Tumorigenic potential of both tumor and normal skin-derived fibroblast cell lines were evaluated using the immunodeficient Rag-2 <sup>-/-</sup> or C.B17-*scid/scid* mice, as described in Herbst et al. [5]. Aliquots of 1–5×10<sup>6</sup> cells suspended in 100 µl PBS were injected into the margin in the pinna and the mice were observed weekly for at least 4 months for evidence of tumor development.

### 2.3. DNA extraction

DNA was prepared from approximately 1×10<sup>7</sup> cells using standard proteinase K digestion followed by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation [7].

### 2.4. RNA extraction

Total RNA was extracted from 3×10<sup>7</sup> cell using a RNA extraction kit (Stratagene, La Jolla, CA, USA; # 200345) as per manufacturer's protocol.

### 2.5. Differential message display RT-PCR

Briefly, the total RNA preparations were treated with RNase-free DNase to remove possible chromosomal DNA contamination. The cDNA was synthesized from the total RNA samples by reverse transcription using 4 sets of degenerate anchored (3') primers (T12MN) where M is G, A, or C and N is G, A, T, or C. With 12 possible combinations of the last 2 bases, each primer recognized 1/12th of the total mRNA population. Partial cDNA sequences were amplified using 5' end primers, corresponding 3' end primers, and <sup>32</sup>S labeled dATP such that 50–100 cDNAs were amplified. The 5' primers were arbitrary decamers allowing annealing positions to be randomly distributed in distance from the polyA tail. The 5' primers were designed to maximally randomize the 3' end with a fixed 5' end. Following amplification, short 100–500 bp cDNA sequences were separated on polyacrylamide sequencing gels. The products

from tumorigenic and normal cells were run on adjacent lanes, allowing side-by-side comparison of the mRNA expression pattern of tumorigenic versus normal skin fibroblasts. Bands that were present in one cell line but absent in the other were cut from the gel, cloned into pGEM easy vector systems (Promega Corporation; Cat# TM042) and sequenced manually.

### 2.6. Northern blotting

Cloned DD-RT-PCR products were <sup>32</sup>P-dTTP labeled and used as probes on Northern blots to verify differential expression. Total RNA samples (30 µg per lane) from matched pairs of cell lines (FP and normal) were run on agarose gels and blotted onto nitrocellulose membranes. Probes were hybridized for 72 h at 42°C and washed in 2× SSC [7].

## 3. Results

The cultured tumor-derived fibroblasts were morphologically indistinguishable from cultured normal fibroblasts under light microscopy as observed previously [5]. The FP-derived fibroblast lines, however, were tumorigenic when injected into the ears of Rag2 <sup>-/-</sup> or *scid/scid* mice whereas the normal fibroblasts did not develop tumors.

DD-RT PCR yielded several cDNA segments that appeared to be either overexpressed or underexpressed in vitro in tumorigenic FP fibroblasts compared to normal fibroblasts (Fig. 1). Of these, four unique overexpressed products (ranging in size from 189 to 412 bp) and three underexpressed products (193–401 bp) were cloned and sequenced.

### 3.1. FP overexpressed transcripts

Positive (sense) strand homologies to expressed sequence tags (ESTs) and short coding regions were found for all of the four products, however, extensive matches of the full length product sequence to 3' cDNA of known genes were found for only two of these products (LHHCM4-5 and LHHCM8-3). Both of these were confirmed by Northern blots to be overexpressed in tumor compared to normal (Fig. 2). The other two clones (LHHCM2-2 and LHHCM7-4) didn't show any homology in the GeneBank and surprisingly their differential expression could not be detected in the Northern blotting experiments in either tumor or normal fibroblast RNA under the conditions used. Repetition of these experiments with excess amounts of RNA will determine if these transcripts are actually low in copy number.

The first 31 nucleotides of product LHHCM8-3 (386 bp) had sense strand homology to the 3' end of pig and human beta-hexosaminidase (X92379.1 and HUMHEXB, respectively), and the putative amino acid sequence was homologous to the 3 terminus of beta-hexosaminidase transcript of the pig (Q29548) and human (P06865). A putative amino acid region of 153 bp long had 38/51 (74%) amino acid matches to the 3' terminus of the pig hexosaminidase mRNA. The remaining portion of product LHH8-3 was a non-peptide sequence.

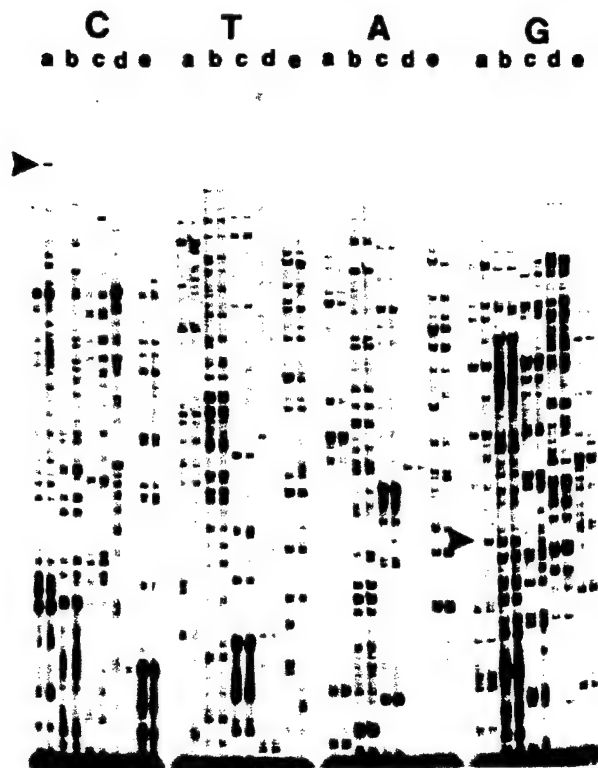


Fig. 1. Gene expression profiles of genes by differential message display studies using total RNA, isolated from a pair of matched tumor and normal skin derived fibroblast cell lines cultured from a turtle with experimentally induced FP. Columns C, T, A, and G represent each of the 3' primers ( $T_{12}CN$  where N = C, T, A, and G, respectively). Subcolumns a, b, c, d, and e represent individual 5' primers (arbitrary decamers). For each of the 20 primer pair combinations, products of normal fibroblasts (left) are displayed along side those from tumor fibroblasts (right). Differentially expressed gene sequences (open and closed arrowheads) were cloned and sequenced for further characterization.

Product LHHCM4-5 (412 bp) was homologous over its entire length to the 3' terminus of eukaryotic peptide (AB029089) and hamster (MAC114, MAC111). Comparison of LHHCM 4-5 with the full-length human cDNA (HS-HCGVII) revealed 4 short gaps of 3, 19, 2, and 23 bp in the sequence alignments and was 88% identical (207/235bp) if the gaps are not considered. Interestingly the putative 3' end of LHHCM 4-5 contained a 130 bp sequence, which was unique. The product is not in the amino acid coding part of the transcript.

### 3.2. FP underexpressed transcripts

Three RT-PCR products that appeared to be relatively underexpressed in FP fibroblasts were also successfully cloned and sequenced. Only one of these clones, LHHCM10-5 (321 bp) was confirmed to be underexpressed in FP by Northern blotting (Fig. 2). This sequence had only limited homology to known mammalian sequences. The longest of the sense strand matches was human and bovine

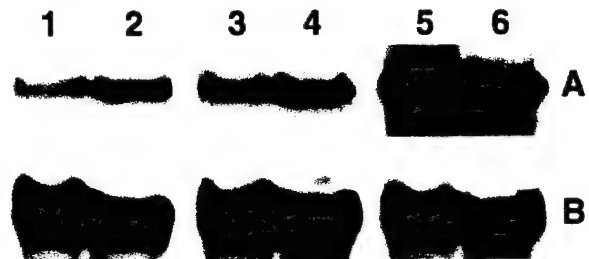


Fig. 2. Northern blotting of total RNA of normal (lanes 1, 3 and 5) and tumor (lanes 2, 4 and 6) cells using three probes derived from the differential product sequences. Lanes 2 and 4 show over-expression of two sequences (LHH4-5 and LHH8-3) and lane 6 shows under-expression of sequence LHH10-5 in the tumor samples. The 18S RNA probe (B) was used to confirm equal loading of RNA samples.

thrombospondin-1 mRNA, which contained a 96 bp sequence that was 83% identical. Sequence LHHCM5-3 (359 bp) had partial sequence homology to chicken delta EF1, a transcriptional repressor (58% base pair identity over 90 bp) and to a region containing human HLA class II gene sequence (68% base pair identity with two gaps of 77 bp and 4 bp, respectively, over 140 bp). This transcript was not detected by Northern blotting in either FP or normal fibroblast RNA under the conditions used, suggesting its low copy number. Product LHHCM1-4 (401 bp) hybridized to RNA from both cell types but was not differentially expressed. This sequence did not have any obvious matches to known genes in the databases.

### 4. Discussion

The availability of matched green turtle FP-derived and normal fibroblast lines and a model system in which to monitor the tumorigenic phenotype made it possible to investigate the molecular basis of fibroblast proliferation in green turtle fibropapillomatosis.

Comparison of gene expression in matched tumorigenic FP-derived versus non-tumorigenic normal skin-derived fibroblasts from individual green turtles with experimentally induced FP yielded several cDNA products by RT-PCR. Two of these were confirmed to be overexpressed in tumor fibroblast RNA and one was confirmed to be underexpressed by Northern blotting. The fact that some of the remaining products could not be detected in Northern blots of either cell type may indicate that these transcripts were very low in copy number. The total RNA was extracted from mass cultured fibroblasts, which could contain an undetermined proportion of tumor cells mixed with normal cells. Therefore, differences in gene expression that were detected by Northern blotting are probably robust differences and more subtle differences will require cloned cell lines.

Very few turtle or reptilian gene sequences have been available in the gene sequence databases. Consequently, all of the partial cDNA sequences found in this study are novel

and cannot be assigned with certainty to their putative corresponding mammalian homologue. In addition, some of the over-expressed products may represent viral gene transcripts from the FP transmissible agent, if it latently infects fibroblasts. Preliminary data from these cell lines suggest that FP fibroblasts are non-productively infected with a green turtle FP-associated herpesvirus, a candidate for the FP etiologic agent [8], so it is possible that some of these sequences could also belong to the herpesvirus genome. For each product, the full-length cDNA must be sequenced and then studied further to characterize putative gene function and to determine whether a corresponding polypeptide is expressed. Extensive additional gene sequence information for green turtles and FP-associated turtle viruses will also be needed before these products can be properly identified and before it can be determined if these products represent normal transcripts whose expression level has changed or aberrant transcripts resulting from gene mutation, activation of pseudogene expression, or abnormal RNA processing.

These results provide a foundation for developing hypotheses about the pathogenesis of fibroblast transformation in FP. For example, the role of polypeptide chain releasing factor or eukaryotic RF1, the putative identity for product LHHCM4-5, in neoplasia or viral infection deserves further investigation. This protein is responsible for chain termination at all 4 stop codons [9]. Overexpression of an aberrant form of this protein, argueably may disrupt an important checkpoint in preventing the translation of abnormal mRNA transcripts, which could enhance cancer progression [10,11].

Product LHH10-5, which is underexpressed in FP fibroblasts, is a putative mammalian homologue of thrombospondin. Thrombospondin is an inhibitor of angiogenesis and a known tumor suppressor [12–14]. Its expression is decreased in a number of neoplastic diseases and in cells infected with human cytomegalovirus, a herpesvirus, as well [15].

The putative identity of LHHCM sequence 8-3, is beta hexosaminidase based on base pair and amino acid homologies to the mammalian gene. Isozymes of beta-hexosaminidase have been shown to be overexpressed in other types of neoplasia [16–21] and increased levels have been detected in the serum of virus infected humans [22]. Thus over expression of this gene is expected in FP. Therefore, isozymes of this protein may serve as useful serum markers for turtles with cryptic FP, such as visceral tumors, or systemic virus infection.

As stated, further elucidation of the role of these genes in FP tumorigenesis or FP-virus infection will require, identification of their full length cDNAs. To establish their respective roles in tumorigenesis will require both in vitro and in vivo studies, such as transfection and overexpression of transcripts in normal fibroblasts to determine if they become tumorigenic. Fortunately, a model system has been developed and matched cell lines are available in our laboratory, which we believe would allow further elucidation of FP in turtles.

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E-mail: bweb@biozentrum.uni-wuerzburg.de

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Department of Medical Genetics  
University of Zürich  
Rämistrasse 74  
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## MOLECULAR MAKERS OF METASTASIS IN DUCTAL MAMMARY CARCINOMA

**P. Mohan R. Achary, H. Zhao, Z. Fan, H. P. Klinger<sup>1</sup>  
and B. Vikram**

Metastasis Laboratory, Departments of Radiation Oncology  
and Molecular Genetics<sup>1</sup>, Albert Einstein College of  
Medicine and Montefiore Medical Center, Bronx, NY.

achary@aecom.yu.edu

About 13% mammary carcinoma patients with negative lymph nodes are prone to developing metastasis even if treated with surgery, or surgery and radiation therapy. The objective of this project is to construct a panel of molecular genetic markers for detecting those 13% of patients so that they could be treated more aggressively.

We hypothesize that in order to achieve the metastatic state primary mammary carcinoma cells must acquire genetic changes in addition to those that led to transformation. A subtractive DNA hybridization technique, Representational Difference Analysis (RDA) was used to compare the DNA of cells from archival normal tissue or primary ductal tumor with that of the metastatic lymph node of the same patient in order to isolate those sequences that were lost in the course of tumor metastasis. The tumor and metastatic cells were recovered by laser capture microdissection and subjected for RDA and also used for Southern blotting and PCR screening with the probes obtained from the RDA procedure.

We isolated 11 sequences that are candidates for being metastasis associated gene sequences (MAGS) because they were lost in metastatic cells. Radiation hybrid (RH) mapping of these sequences agreed with the results of sequence homology localization searches. To-date three of these 11 sequences were used to screen normal, primary and metastatic cell DNA samples. MAGS-XI was found to be lost in the metastatic cells of 3 out of the 5 tumors. MAGS-IX was found to be lost in metastases from 2 out of 3 primary tumors, and MAGS-IV was lost in 1 out of 3 tumors. RH mapping and homology search results indicated that MAGS-IX was located on the long arm of chromosome 10 where the PTEN, a known metastasis suppressor gene is also located. To determine if MAGS-IX is perhaps a part of the PTEN gene we PCR screened the above mentioned three tumor cell DNA samples and a breast carcinoma cell line, HCC-1937 which has homozygous loss of the PTEN gene (10q23 region). The results indicated that MAGS-IX is not related to the PTEN gene but is a novel gene sequence. Presently we are isolating partial and/or full-length sequences of these MAGS to use as fluorescence *in situ* hybridization (FISH) probes to screen a larger number of tumor samples. A 2Kb sized MAGS-IX has been generated and localized to the q21 region of human chromosome number 10 by FISH (Figure). Screening the primary tumor tissue sections of carcinomas which metastasize to lymph nodes is underway. The panel of molecular markers that we expect to develop should make it possible to detect those tumors (~13%) that are prone to becoming metastatic.

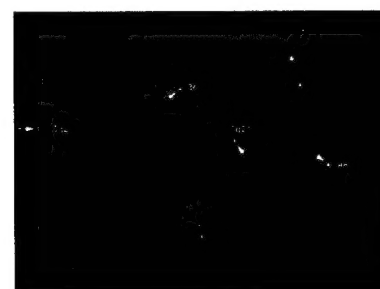


Fig. Localization of MAGS-IX to human metaphase chromosome 10q21 region by FISH. Centromere of chromosome 8 was used as a positive control probe. Both sequences were labeled with spectrum green (Vysis) and chromosomes were counter stained by DAPI.

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